PHYSIOLOGICAL ONTOGENY IN THE TOBACCO PLANT

THE EFFECT OF VARYING WATER SUPPLY ON THE DRIFTS IN DRY WEIGHT AND LEAF AREA AND ON VARIOUS COMPONENTS OF THE LEAVES

by the late A. H. K. PETRIE AND J. I. ARTHUR
(compiled by J. G. WOOD)

(From the Waite Agricultural Research Institute, University of Adelaide).

(Accepted for publication 13th September, 1943.)

The experiment recorded below was designed by the late Dr. A. H. K. Petrie to investigate the effect of varying water supply on the quality of leaf produced by the tobacco plant. The analytical work was carried out entirely by the second named author and was not completed until after the death of Dr. Petrie. It was the intention of the latter to make detailed analyses of growth rates and of material interchanges among different leaf groups. The compiler has restricted the present paper to an interpretation of the effects of varying water regimes on the total dry weight increase. Records of the analytical work on the separate leaf groups are available to persons interested and may be obtained on request to the Director of the Waite Institute.

EXPERIMENTAL PROCEDURE.

Culture.

On 26th September, 1939, seeds of *Nicotiana tabacum* L. var. Hickory Prior were sown in soil and on day 51 were planted out: one seedling in each of 204 pots containing 14 kg. of a mixture of two-thirds water-washed sand and one-third Waite Institute loam. The pots were randomized in a glass house within 6 blocks according to the time and treatment planned for them. Each pot received the following three nutrients in solution: $NaNO_3$, 9·11 gm. per pot; K_2SO_4, 5·57 gm. per pot; $Ca(H_2PO_4)_2.2H_2O$, 2·44 gm. per pot. The first two solutions were applied on day 50 and the third on day 55.

Treatment.

Water regime. The pots were further subdivided into four groups for the following treatments:

Treatment I. *High-water range*, i.e. above the moisture equivalent throughout the experiment. The water content of the soil was maintained between the extremes 12·76–27·76 p.c. (1,694–3,685 ml. water per pot).

Treatment II. *Low-water range.* The extremes were wilting point and moisture equivalent, these being 4·3–7·76 p.c. (571–1,030 ml. water per pot). Treatment commenced on day 58 and continued throughout the experiment.

Treatment III. *Early temporary drought.* The water percentage was reduced to 6·97 p.c. Treatment commenced on day 74 and restored to level of Treatment I on day 81.

Treatment IV. *Late temporary drought.* The water percentage was reduced to 3·22 p.c. Treatment commenced on day 100 and restored to level of Treatment I on day 112.

The moisture content of the soils was controlled by weighing the pots at suitable intervals, allowance being made for the weight of the plants. Evaporation was restricted by spreading 1 kg. of fine gravel on the surface of each pot.

Topping. With the exception of half the plants of Treatments I and II all plants were topped, plants of Treatments I, III and IV on day 98 and those of Treatment II on day 106. The plants were topped immediately above leaf 21.

As axillary buds or suckers developed they were removed, dried and stored.

Harvests.

Harvests were made as follows: 1, day 34; 2, day 41; 3, day 49; 4, day 73; 5, day 85; 6, day 99; 7, days 118–119; 8, days 146–147; 9, untopped day 168, remaining plants days 174–175. As plants of Treatment II reached maturity at a later date than those of the other treatments a further harvest of these plants (Harvest 10) was made on day 231. Harvests 1, 2 and 3 were made by thinning the plants, 6 being selected at each harvest. The remaining harvests were

made from the appropriate number of pots, so that six replicates, one from each block, were obtained for each treatment.

Separation of Parts.

This was carried out as described previously by Petrie, Watson and Ward (1939).

The leaves were divided into four groups: Group 1 (lower): leaves up to and including leaf 11. Group 2 (lower middle): leaves 12–17 inclusive. Group 3 (upper middle): leaves 18–21 inclusive. Group 4 (upper): all leaves above leaf 21 in untopped plants.

Analytical Methods.

Total N was estimated by the micro-Kjeldahl method on the ground dried material after reduction of the nitrate present.

Protein-N was estimated by micro-Kjeldahl on the residue obtained by grinding the dried material with an acetate-acetic acid buffer at pH 4·05 containing 40 p.c. alcohol, heating at 80° C. for 10 minutes and filtering at a temperature below 5° C.

Reducing sugars and sucrose were determined on fresh leaf material by the procedure described by Amos and Wood (1939) and reported as hexose.

"Labile reserves" and crude fibre. Residues from the alcoholic extraction of sugars were refluxed with 1·5 p.c. H_2SO_4 and the reducing power of aliquots of the hydrolysate, before and after fermentation with yeast, was determined by the method of Hagedorn-Jensen. The hexose equivalent was taken as a measure of labile reserves. The residue from the acid extraction, after boiling for ½ hour with 1·25 p.c. KOH, was filtered, dried and weighed to give an estimate of the amount of crude fibre.

All estimations were carried out on duplicate samples of the original material and the mean values reported. All values are reported in grams or in the case of relative values as grams associated with 100 gm. dry matter.

DISCUSSION.

Dry Weight Data.

Total plant. Values for total dry weight plotted against time, together with significant differences derived from analyses of variance are given in Fig. 1. Well marked effects of treatment are shown.

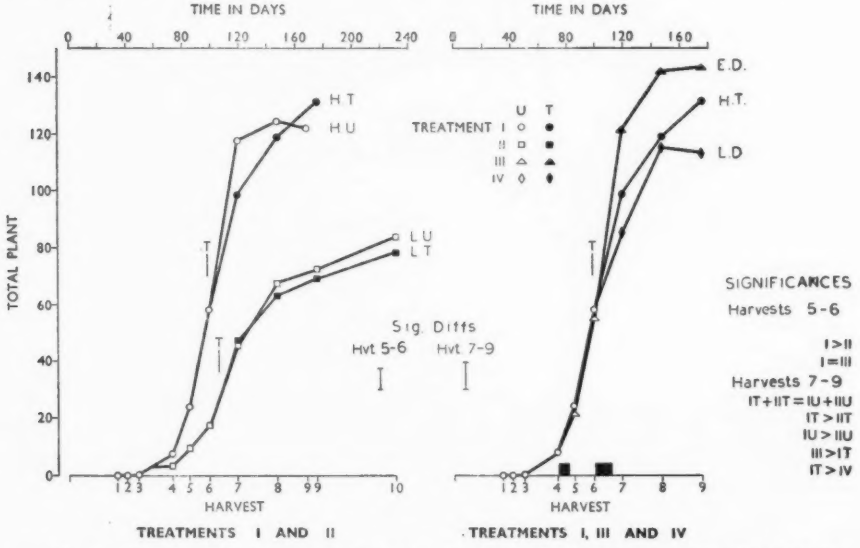


Fig. 1. Dry weight of total plant (gm.). In this and subsequent figures the following abbreviations are used to aid identification of the relevant curves: H.T., high water range, topped (Treatment I); H.U., high-water range, untopped (Treatment I); L.T., low-water range, topped (Treatment II); L.U., low-water range, untopped (Treatment II); E.D., early temporary drought (Treatment III); L.D., late temporary drought (Treatment IV). T indicates the time of topping.

Compared with high-water treatment there is a depression in dry weight at all harvests under continuous low-water treatment, together with a longer period of growth. The latter effect is illustrated in Fig. 2 where the time of ripening (Munsell YGY 7/8 at end and GY 7/8 at bottom) of each leaf in the different treatments is recorded.

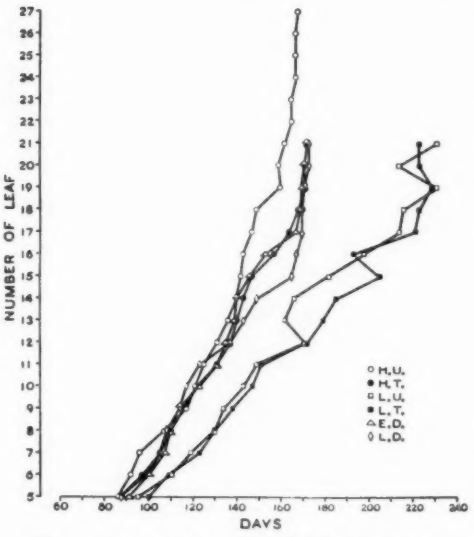


Fig. 2. Times of ripening of individual leaves. The leaf number refers to the order of appearance of the leaves; the time axis denotes days after planting.

Early temporary drought causes first an insignificant depression in dry weight followed by a significant increase.

Late temporary drought causes an initial depression which is significant. The late drought was applied at time of maximum dry weight of the leaves and resulted in more rapid senescence and loss of dry weight than in the high-water plants, especially in the lowest group of leaves (see Fig. 4).

Topping has decreased the dry weight compared with untopped plants, but the decrease is insignificant, except at Harvest 7 in Treatment I. This result is contrary to that recorded previously for tobacco by Petrie, Watson and Ward (1939) and is discussed in the next section.

Main parts. Dry weights of leaves, stems, roots and inflorescence in different treatments plotted against time are given in Fig. 3. Results of analyses of variance of leaf dry weights are also given.

Treatment effects are similar to those described for the total plant with one important difference, viz. that topping has increased the weights of all parts, except in the stems at Harvest 7. The opposite effect due to topping seen in the data for the whole plant is accounted for by the inflorescence whose dry weight has increased more than that of the parts of the topped plants. In the experiment described by Petrie, Watson and Ward (1939) the inflorescence was less effective in causing loss of dry weight from other parts, being less than 10 p.c. of the total plant weight, whereas in the experiment described here the inflorescence comprises approximately 30 p.c. of the total plant weight. The increased weight of leaves, stem and root in topped plants tends to be the counterpart of the inflorescence in the untopped plants in Treatments I and III.

TABLE 1.

Crude fibre in leaves as percentage of dry weight of leaves.

Harvest Treatment	1	2	3	4	5	6	7	8	9
I (untopped)	4·2	3·83	5·21	3·51	3·73	4·12	3·70	5·46	5·20
I (topped)							3·58	3·91	4·46
II (untopped)				3·15	3·90	4·33	4·17	5·70	5·65
II (topped)							3·93	4·90	4·60
III					4·18	4·79	4·07	4·24	4·22
IV							4·74	4·43	4·64

Except in the untopped series of Treatment I, a senescent decline in total dry weight of leaves does not occur. Examination of the dry weights of the four leaf groups in each treatment (Fig. 4) reveals that whilst the untopped plants in Treatment I exhibit such a decline in all leaf groups, in other treatments the decrease in dry weight obvious in the lowest leaf group is more than compensated for by the increase in dry weight of the higher leaf groups.

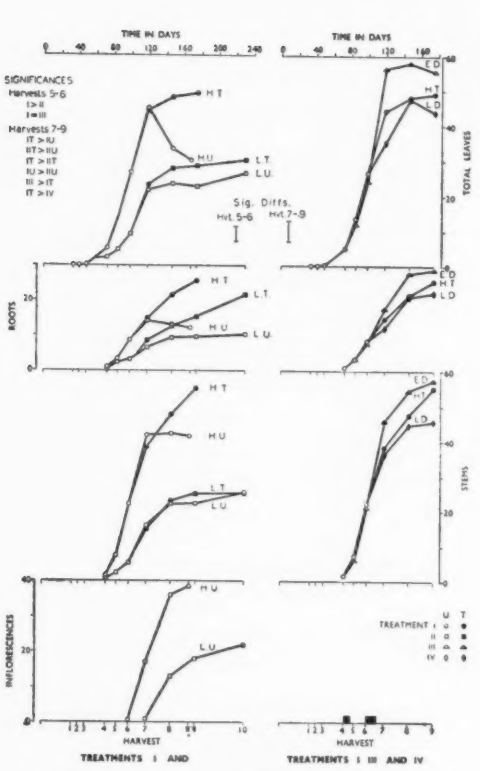


Fig. 3. Dry weight of parts (gm.).

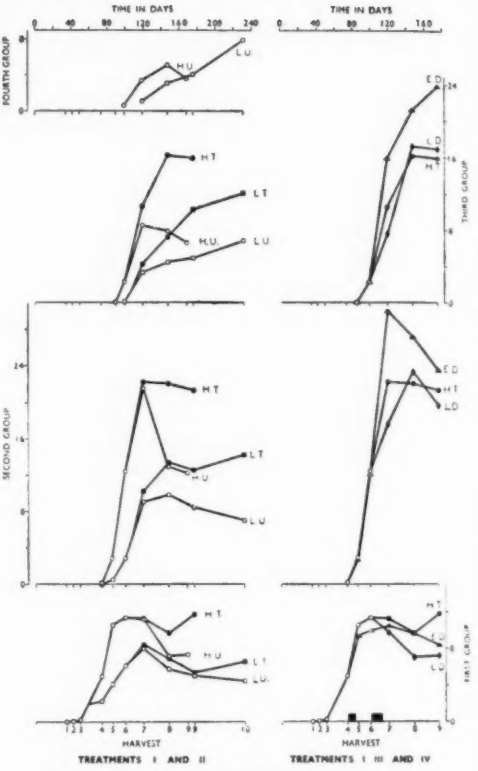


Fig. 4. Dry weights of leaf groups (gm.).

Drifts of Attributes of the Leaves.

Crude fibre and labile reserves. Absolute amounts of crude fibre in the leaves are plotted against time in Fig. 5. In general, the effect of treatment on leaf fibre content is similar to that in total dry weight. Permanent drought at all harvests results in lower fibre content than in fully watered plants. Early drought causes first a depression in fibre content followed by an increase compared with the high-water series. Topping also results in increase in amount of fibre.

On a relative basis (percentage of total leaf dry weight, see Table 1) crude fibre of topped plants is less than that of untopped plants owing to greater loss of dry weight from leaves of untopped plants during senescence. Low-water and both early and late drought result in increase in percentage crude fibre compared with the controls, although the increase is small.

Absolute amounts of labile reserves (Fig. 6) present a similar picture to that of crude fibre so far as treatment effects are concerned, but decrease in amount of these reserves owing to translocation to other sinks during senescence is marked.

The general picture which emerges from examination of these carbohydrates is a marked effect of treatment on amount of photosynthesis. Compared with the normal, high-water plants, permanent drought causes a depression in amount of photosynthetic products, both early and late droughts cause an initial depression followed by increase and topping causes an increase.

Protein-nitrogen and total-nitrogen. Absolute protein-N of the leaves is plotted against time in Fig. 7. A marked decrease in protein content occurs during senescence similar to that previously discussed by Watson and Petrie (1940). Treatment has a marked effect upon protein content. Compared with untopped plants, topping increases amount of protein and both early and late drought bring about an increase in amount of protein compared with controls.

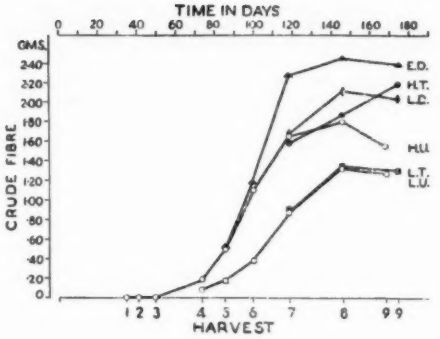


Fig. 5.

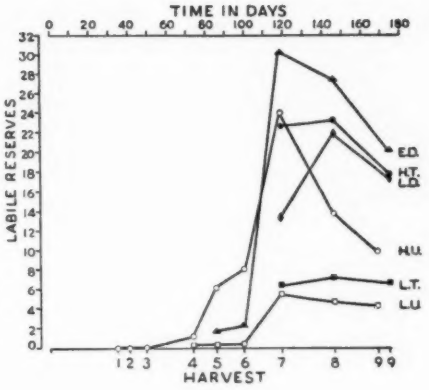


Fig.6.

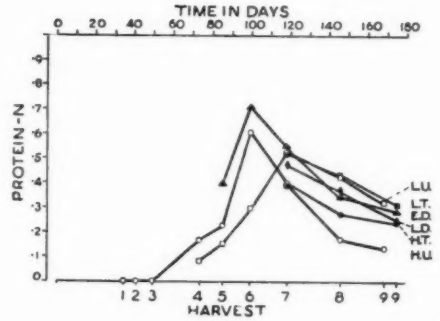


Fig. 7.

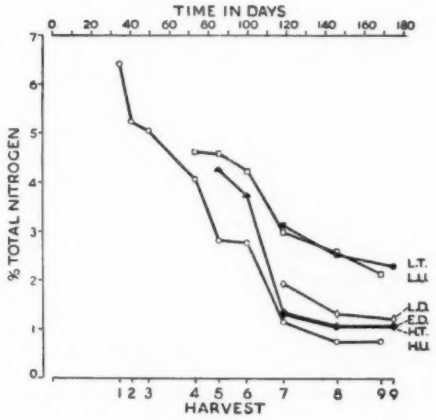


Fig. 8.

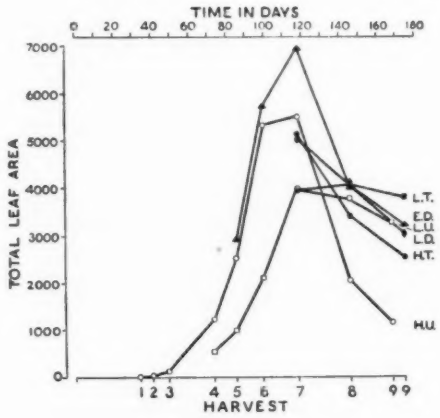


Fig. 9.

Fig. 5. Absolute amount of crude fibre in leaves (gm.).

Fig. 6. Absolute amount of labile reserves in leaves (gm.).

Fig. 7. Absolute amount of protein in leaves (gm.).

Fig. 8. Total nitrogen as percentage of dry weight of leaves.

Fig. 9. Area of leaves (sq. cm.).

In plants subjected to permanent drought the total protein content is less than that of high-water plants at harvests up to Harvest 7, but the maximum protein content of low-water plants, although attained later than that of high-water plants, is not very different from that of the maximum content of the high-water plants.

During senescence the protein content of all treated plants is higher than that of the untopped high-water plants owing to removal of sinks in topped plants and the prolonged growth cycle of the low-water plants.

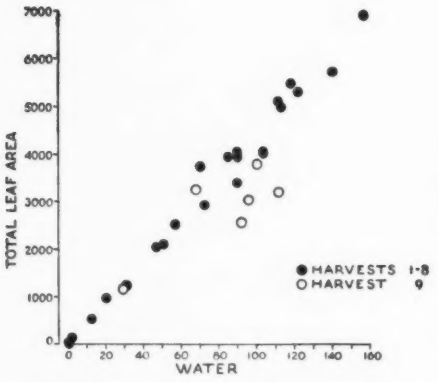


Fig. 10a. Leaf area plotted against absolute amount of water in leaves.

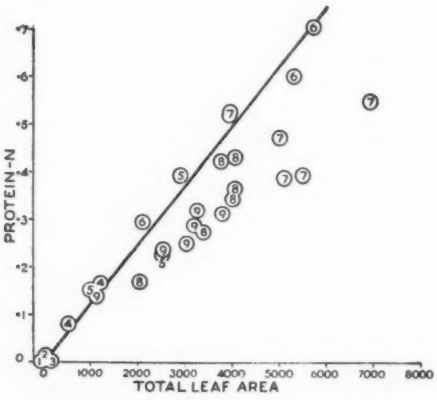


Fig. 10b. Leaf area plotted against absolute amount of protein in leaves. The numbers in circles refer to the harvest.

Absolute total-N plotted against time is not figured but shows the same trends as does protein-N. Total-N as percentage of dry weight is plotted against time in Fig. 8. Protein-N as percentage dry weight shows similar trends and relative values to those of total-N. It is apparent that low-water plants possess a higher relative amount of protein than the high-water plants; and also that in plants subjected to temporary drought at harvests following the drought period the relative protein content is much higher than in the control plants; at the later harvests the relative protein content of plants subjected to temporary drought is probably not significantly higher than that of the topped controls.

It would appear therefore that low water content results in a greater uptake of nitrogen from the soil. In plants subjected to temporary drought and then returned to high-water content the additional nitrogen taken up is built up into protein, so that the total protein content is greater than that of the controls. The lower protein content of plants subjected to permanent drought and the later attainment of a maximum protein content approximately the same as that of the high-water plants suggests that rate of production of carbohydrates may be limiting and this is borne out by examination of Fig. 6 which shows that labile reserves in low-water plants remain at a very low level until Harvest 6.

Leaf area. The curves for total leaf-area/time (Fig. 9) resemble the curves for protein/time. Unlike the leaf dry weight but like that of leaf protein, a senescent decline in leaf area is obvious in all treatments. Within the whole data in all treatments, with the exception of Harvest 9, leaf area shows a high linear correlation with absolute water content of the leaves (Fig. 10a); up to the time of maximum area of the leaves, leaf area also shows a high linear correlation with absolute protein content (Fig. 10b), but after Harvest 6 other factors than protein determine leaf area; up to the time of maximum leaf area (Harvest 6) water content shows a high correlation with protein content.

DERIVED QUANTITIES.

Before attempting to interpret the dry weight data in terms of secondary quantities derived from the primary data, the compiler considers that discussion on the most suitable basis on which to express these quantities is pertinent.

The best measure of growth is the amount of energy fixed from the universe, and Long (1934) has shown that dry weight of a plant is an adequate measure of calorific value.

Recently Larsen (1942) has shown the similarity of the dry weight/time curve directly determined with that calculated from the difference between assimilation of the leaves and respiration rate of the whole plant measured at intervals during the life cycle. The latter quantity is the Unit Leaf Rate of Briggs (1920).

Measurement of rate of photosynthesis is best expressed on the basis of amount of energy absorbed. Leaf dry weight is an unsuitable basis for expression since it is equivalent to a variable fraction of the total energy absorbed. Under conditions of constant light intensity the amount of energy absorbed will depend upon the area of the leaf and the amount of photosynthetic apparatus present. The best measure of the latter available is the total protein content of the leaves (i.e. amount of chloroplast *plus* cytoplasmic component). In the experiment described here there is a high linear correlation between leaf protein content and leaf area up to the time of maximum leaf area (*vide* Fig. 10b). Up to this time therefore both leaf area and protein content are adequate measures of the energy absorbed, provided climatic factors remain constant. But over the whole data for the experiment leaf area shows a high correlation with leaf water content and after attainment of maximum area leaf area decreases less rapidly than does leaf protein. During this senescent phase an increasing number of leaves ripen, i.e. become yellow-green and finally yellow (*vide* Fig. 2). During this stage of the life cycle leaf area no longer gives an adequate measure of the energy absorbed; a better measure will be given by protein content, although this, at best, is only an approximation, for Wood (1941) has shown that at full yellow stage of leaves all chloroplast protein has been hydrolized but a residue of cytoplasmic protein remains.

For these reasons we prefer to express assimilation rate on a leaf-protein basis (P).

There remains for consideration the other component of Net Assimilation Rate $\left(\frac{dW}{dt\ P}\right)$ viz. respiration on a protein basis. The data of Gregory and Sen (1937), Richards (1938), Petrie and Williams (1938) and Wood (1941) indicate that in plants with excess of nitrogen nutrient respiration rate on a protein basis (OR_p) remains approximately constant throughout the life cycle, (i.e. $\frac{dW}{dt\ P}=\frac{\text{assim. rate}}{P} - K$). When nitrogen nutrients are deficient OR_p increases in an approximately linear manner with time, fluctuations in rate being caused by fluctuations in amino-acid and sucrose contents (i.e. $\frac{dW}{dt\ P}=\frac{\text{assim. rate}}{P} - Kt$). Investigation of the data of Larsen (1942) shows that unit leaf rate on an area basis takes the form $\frac{dW}{dt\ A}=\frac{\text{assim. rate}}{A}(1 - Kt)$.

Taking the most complex of these relations it appears that expression on a protein basis permits approximate evaluation of unit leaf rate in terms of assimilation rate per unit of energy absorbed and a linear function of time, the interpretation of which remains obscure but which may be due to differences with increasing age of storage and protoplasmic proteins, in the nature of oxidizing systems or in the nature of substrates. We have therefore used net assimilation rate on a protein basis in interpreting the dry weight curves.

The growth rate $\left(\frac{dW}{dt}\right)$ is the product of Net Assimilation Rate on a protein basis and the total leaf protein. Relative Growth Rate $\left(\frac{dW}{dt\ W}\right)$ for higher plants

does not possess the significance it has for lower organisms (as yeast), where W is then a measure of the actual growing material. It is a convenient measure for rates of increase in dry weight at different times in different plants and gives correctly the average values for relative growth rate over the period between two observations. The latter property is somewhat nullified by the fact that in analysis of relative growth rate into components, assimilation rate on the basis of some leaf attribute $\left(\frac{dW}{dt\ L}\right)$ is calculated from the approximate formula $\frac{W_2-W_1}{t_2-t_1}\cdot\frac{\log L_2-\log L_1}{L_2-L_1}$ where W_2 and W_1 are total dry weights and L_2 and L_1 measures of some leaf attribute at the times t_2 and t_1.

Another advantage of Relative Growth Rate in analysis of growth is that it allows this quantity to be divided into other easily grasped parts, e.g. $\frac{dW}{dt\ W}=\frac{dW}{dt\ P}\cdot\frac{P}{W_L}\cdot\frac{W_L}{W}$, that is, the Relative Growth Rate is equal to the product of the net assimilation rate on a protein basis, the amount of leaf protein per unit of leaf dry weight and the leaf dry weight as percentage of the total dry weight.

On the other hand Relative Growth Rate possesses disadvantages in the analysis of growth data. For example in the present experiment, comparing the low- and high-water plants in early harvests, $\frac{dW}{dt\ P}$ is greater in high-water plants than in low, whilst $\frac{P}{W_L}$ is greater in low-water plants than in high-water plants and $\frac{W_L}{W}$ is approximately constant in both series. Under these circumstances the Relative Growth Rates are approximately constant in the two series, although growth rates are markedly different. By the use of Relative Growth Rate therefore the cause of greater absolute dry weight in one treatment is obscured.

For these reasons, and in spite of the rather long intervals between some harvests, the compiler has preferred to interpret the dry weight data in terms of the first differential $\frac{dW}{dt}$ and to analyse this into the components, net assimilation rate on a leaf-protein basis and total leaf protein.

Net Assimilation Rate.

Curves for net assimilation rates (unit leaf rates) on dry weight, area and protein bases, plotted against time, together with data for mean maximum daily temperature are given in Fig. 11. The trends of the ratio on leaf area and on leaf protein basis are similar, as might be expected from the correlation between protein content and leaf area over much of the life cycle. On a dry weight basis the trend is different; obviously the trend would be similar, say to those on a protein basis only if the amount of protein per unit of leaf dry weight were constant. For the reasons given above attention has been restricted to net assimilation rate on a leaf protein basis.

Since net assimilation rate on a protein basis is a function of assimilation rate, it might be expected that this ratio would be dependent on climatic factors and especially on temperature (*vide* Williams, 1939). Such dependence is not apparent from the data (Fig. 11) since during the period of early harvests mean temperature remained approximately constant and the rise in temperature towards the end of the life cycle of the plants occurred when other factors, presumably internal as discussed previously, caused a decrease in net assimilation rate.

During Harvests 1–7, net assimilation rate on a protein basis (E_p) of the high-water plants is greater than that of plants subjected to permanent drought; early drought causes an initial depression in E_p to a value approximately the same as that of the low-water plants. Late drought also causes an initial depression in E_p. In both cases when plants subjected to temporary drought are returned to high-water content the value of E_p returns to a value approximately the same as that of the controls.

Tiver (1942) has also shown that low-water treatment causes depression in net assimilation rate on a protein basis in the flax plant. The reason for the decrease in net assimilation rate in plants subjected to low-water content is not clear. Dastur (1925) has reported a high correlation between rate of photosynthesis and absolute water content of leaves. Over the period of the experiment discussed (Harvests 1–7) water content shows a high linear correlation with protein content over the whole range of treatments, i.e. the amount of water associated with unit of protein is approximately constant. It is unlikely that water exerts an effect *per se*. It is possible that the amount and consequently the concentration of an inhibiting substance, not measured here, is increased by low-water treatment, or that the amount of a reactant, enzymatic or otherwise, is decreased in amount.

Whilst therefore the mechanism remains obscure it is apparent that permanent or temporary drought reduces net assimilation rate to a value lower than that of fully-watered plants. Superimposed upon the effect of low-water treatment, there is a decrease

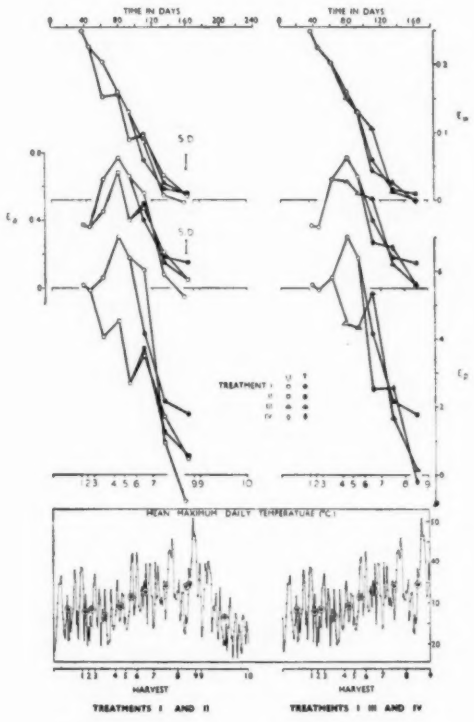


Fig. 11. Net assimilation rates (E_w on dry weight basis; E_a on area basis; E_p on protein basis) and mean maximum daily temperatures.

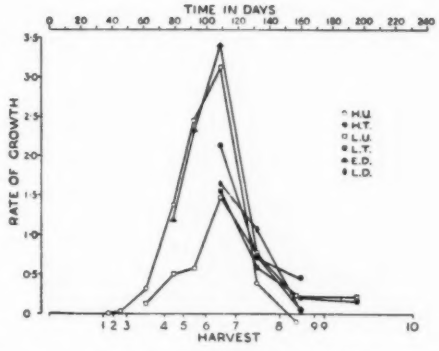


Fig. 12. Growth rates of total plant.

in E_p with time, especially during the senescent phase, e.g. the value of E_p for low-water treatment decreases progressively with time although the water content per unit of protein remains approximately constant. Part of this decrease in rate is undoubtedly due to the loss of chloroplasts described earlier, but other components of the photosynthetic system may also decrease in amount during senescence. During the late harvests it is noteworthy that in the topped plants, as well as in the low-water plants, where senescence is less rapid and the protein content is higher, net assimilation rate is higher than in the untopped plants with normal water regime.

Growth Rate.

Growth rate has been calculated from the formula $\frac{W_2 - W_1}{t_2 - t_1}$ where W_2 and W_1 are the dry weights at times t_2 and t_1. The curve for growth rate plotted against time is given in Fig. 12.

Up to the interharvest period 6–7, the growth rate of plants subjected to permanent drought is less than that of high-water plants, for in the latter both net assimilation rate on a protein basis and leaf protein content are greater than in the former. After harvests 6–7 the growth rate of low-water plants is greater than that of the controls since the net assimilation is approximately

the same in both treatments but the protein content, due to delayed senescence, is greater in the low-water plants than in the high-water plants.

Both early and temporary drought cause an initial depression in growth rate due to a depression in net assimilation rate; this is followed by an increase in growth rate greater than that of the high-water plants. This increase is due to the greater protein content of the plants subjected to temporary drought.

CONCLUSIONS.

The growth curves are explicable in terms of net assimilation rate on a protein basis and of protein content.

It is shown that when tobacco plants are subjected to drought, either permanent or temporary, compared with fully-watered plants the net assimilation rate on a protein basis is decreased and at the same time there is a greater percentage uptake of nitrogen from the soil.

When plants subjected to temporary drought are returned to high-water conditions, the net assimilation rate is restored to approximately the same value as that of the control high-water plants but with return of the high assimilation rate the protein content of the leaves becomes greater than that of the controls and the growth rate consequently becomes greater than that of the controls.

In plants subjected to permanent drought, the net assimilation rate is considerably lower than that of the fully-watered plants and since the maximum protein content of the leaves is attained later than that of the fully-watered plants, carbohydrate content may limit protein formation. The slower growth rate delays senescence with its accompanying loss of leaf protein so that during late harvests the growth rate of the low-water plants is greater than that of the controls.

REFERENCES.

Amos, G. L. and Wood, J. G. (1939): Austral. J. exp. Biol., 17, p. 285.
Briggs, G. E., Kidd, F. and West, C. (1920): Ann. Appl. Biol., 7, p. 202.
Gregory, F. G. and Sen, P. K. (1937): Ann. Bot. N.S., 1, p. 521.
Larsen, P. (1942): Planta, 32, p. 343.
Long, F. L. (1934): Plant Physiol., 9, p. 323.
Petrie, A. H. K., Watson, R. and Ward, E. D. (1939): Austral. J. exp. Biol., 17, p. 93.
Petrie, A. H. K. and Williams, R. F. (1939): *Ibid.*, 16, p. 347
Richards, F J. (1938): Ann. Bot. N.S., 2, p. 491.
Tiver, N. S. (1942): Austral. J. exp. Biol., 20, p. 149.
Watson, R. and Petrie, A. H. K. (1940): *Ibid.*, 18, p. 313.
Williams, R. F. (1939): *Ibid.*, 17, p. 123.
Wood, J. G. (1941): *Ibid.*, 19, p. 313.

STUDIES OF THE FLAX PLANT

2. THE EFFECT OF ARTIFICIAL DROUGHT ON GROWTH AND OIL PRODUCTION IN A LINSEED VARIETY

by N. S. TIVER AND R. F. WILLIAMS

(From the Waite Agricultural Research Institute, University of Adelaide).

(Accepted for publication 17th September, 1943.)

In the first paper of this series, Tiver (1942) studied the developmental anatomy of the fibre bundles of flax, and the effects of artificial drought on growth and fibre production. In this second paper a parallel experiment with a linseed variety is described, and the growth responses of the two varieties are compared. The effects of artificial drought applied from the time of commencement of flowering tend to be similar to the effects of delayed sowing in the field, for, as cultivated in Australia, both flax and linseed mature when temperatures are rising and rainfall is decreasing. Thus, while it is realized that the effects of variation of sowing date on the yield of both fibre and oil may be dependent on many other factors such as the extent and depth of the root system, it is contended that the present experiments are relevant to the interpretation of this type of field experiment.

The treatment of the physiology of growth is essentially the same as in the first paper, but the interpretation of treatment effects upon oil yield could not be carried as far as was possible in the case of fibre yield in Liral Crown flax.

SYMBOLS.

E_W Net assimilation rate on a dry weight basis.
E_P Net assimilation rate on a protein-nitrogen basis.

EXPERIMENTAL PROCEDURE.

On May 6, 1941, thirty seeds of linseed (*Linum usitatissimum* L. variety Punjab) were sown in each of 78 pots. Pot culture technique, basal manuring (3 gm. superphosphate per pot) and water treatments were as described for flax in the first paper of this series (Tiver, 1942). The available nitrogen in the soil-sand mixture used for both experiments was probably considerable, since the soil was taken from the surface after a period of bare fallow. Prescott and Piper (1930) have shown that the nitrate-nitrogen content to a depth of 3·5 inches of the same soil type may be as high as 70 parts per million. Knowing the surface area of the pots and the weight of soil used, this may be shown to be equivalent to a dressing of 140 lb. of nitrogen per acre.

Harvests were made on June 18, July 9 and 30, August 12 and 29, September 23, October 22, and November 6. For the first four harvests the dates are identical with those of the flax experiment, but thereafter they differ; harvest 5 was 3 days earlier, and harvests 6, 7 and 8 were 4, 8 and 6 days later respectively in the linseed experiment.

Although the low moisture treatment applied after flowering was essentially the same in both experiments, the transpiration losses per pot were much greater for linseed. Thus the soil moisture was more frequently reduced to the permanent wilting percentage between each harvest (see Figs. 1 and 2). It is thought, however, that the total period of subjection to any given range of water content would be approximately the same in each case.

Six pots, three from each treatment, were set aside for oil estimations and were harvested on November 6; the air-dry weights of the inflorescences and cleaned seed being recorded. These seed samples were extracted with ether in two stages, the samples being reground and mixed with acid-washed sand after partial extraction. The total period of extraction was eleven hours, and the extract was weighed after drying for 1·5 hours at 107° C. The oil content so determined was expressed as a percentage of the air-dry seed.

Data for total-nitrogen, protein-nitrogen and sugars are presented in the appendix. Total nitrogen was determined by the Kjeldahl method. The tannic acid method (Petrie, 1937) was used for the estimation of protein nitrogen in flax leaves, but the method proved unsatisfactory in the case of linseed. The latter material was therefore ground with a mixture of equal volumes of 80 p.c. alcohol and sodium acetate-acetic acid buffer (pH 4·05), heated at 80° C. for ten

minutes, filtered at a temperature below 5° C. and nitrogen determined in the residue. In the absence of interfering substances (possibly mucilages in this instance), coagulation of the proteins is satisfactory with tannic acid, and results by the two methods are in good agreement. Total sugars (sucrose plus reducing sugars) were estimated by a modification of the Hagedorn-Jensen method for blood sugar. Details of the extraction from fresh plant material and also of the correction for non-fermentable reducing substances are given by Amos and Wood (1939).

GROWTH OF THE PLANT AND ITS PARTS.

The basic growth data of the experiment are presented in Table 1 and the dry weights of the plant parts are plotted additively in the upper portion of Fig. 1. At the final harvest, only inflorescence weights were recorded, but, since the plants were practically mature on the previous occasion of sampling (harvest 7), the diagrams of Fig. 1 were completed on the assumption that leaf, stem and root weights remained unchanged over the final harvest interval. The maturity of the plants throughout this interval is indicated by the constancy of inflorescence weights; there are, however, slight increases in seed weights over the same period (Table 3). The form of the growth curve for Punjab linseed with high moisture treatment is similar to that for Liral Crown flax (Tiver, 1942), but the discontinuity induced by the low moisture treatment is less evident in the former. The relative reduction in final yield with this treatment is nevertheless greater for the linseed variety.

TABLE 1.

Dry weights in mg. per plant.

Harvest.	Days from sowing.	Plants per pot (range).	Leaves.	Stems.	Inflorescence.	Roots.	Total.
1	43	30	44·5	23·9	—	27·9	96·3
2	64	29–30	119·6	98·1	—	65·3	283·0
3	85	28–30	221·6	276·9	—	106·8	605·3
4	98	30	311·0	501·9	—	192·1	1,005·0
5H	115	29–30	462·8	861·2	43·2	196·8	1,564·0
6H	140	30	421·9	1,173·5	831·0	404·3	2,830·7
7H	169	29–30	347·0	1,096·5	1,226·8	319·3	2,989·6
8H	184	29–30	—	—	1,229·3	—	—
5L	115	28–31	430·9	779·7	33·6	190·1	1,434·3
6L	140	29–31	397·6	839·9	467·3	239·0	1,943·8
7L	169	30	327·2	797·7	709·1	198·6	2,032·6
8L	184	29–30	—	—	710·5	—	—

H = high moisture. L = low moisture.

Effects of Time and Treatment (Fig. 1).

Although the form of the growth curves is so similar for the flax and linseed varieties, there are striking differences in the distribution of dry matter between the various plant parts (see the lower portion of Fig. 1 in this and in the first paper of the series). The numerical values for the weight ratios at maturity (harvest 7) are shown in Table 2. The varietal differences, which are very apparent in this Table, are without doubt attributable to selection for oil and fibre yield respectively.

TABLE 2.

Weight ratios of plant parts to total plant at harvest 7.

Treatment.	Variety.	Weight ratio. Leaf.	Stem.	Inflor.	Root.
H	Punjab	·116	·367	·410	·107
	Liral Crown	·059	·653	·226	·062
L	Punjab	·161	·392	·349	·098
	Liral Crown	·111	·670	·146	·073

H = high moisture. L = low moisture.

Although Punjab linseed has small leaf and root weight ratios at maturity, they are nevertheless much greater than the corresponding values for Liral Crown flax. The greater values for leaf weight ratio, not only at harvest 7 but at all harvests after flowering, provide a clue to the

differential effects of the moisture treatments upon total yield. With adequate soil moisture, the percentages of the total yield produced after harvest 4 are 66 and 57 for the linseed and flax varieties respectively; a difference clearly attributable to the above-mentioned differences in leaf weight ratio. It is thus not surprising that the low moisture treatment, which was applied over the same period and which drastically limited the synthesis of carbohydrates should more adversely affect the final yield of the linseed variety.

The growth curves for the root systems of linseed, especially with high moisture treatment, exhibit pronounced maxima at harvest 6, and in this respect resemble the behaviour of wheat and oats rather than of flax and tobacco.[1] In spite of the fact that secondary thickening occurs

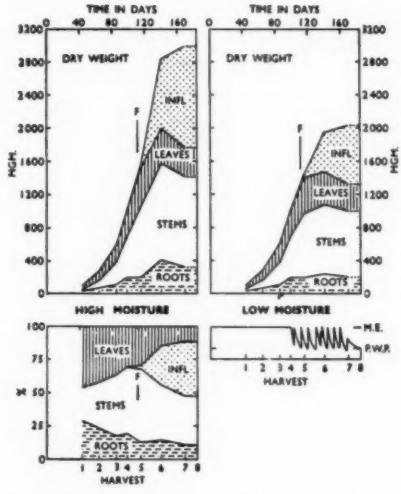


Fig. 1. The two upper diagrams show the dry weights of plant parts plotted additively, and illustrate the effect of the soil-moisture treatments on the growth of linseed. Below, on the left, the relative proportions of the plant parts are shown for the high moisture treatment. Below, on the right, is a diagram representing the variation in soil-moisture content (between the moisture equivalent, M.E. and the permanent wilting percentage, P.W.P.) for pots of the low moisture series. The vertical lines marked F in this and the next Fig. indicate the time of commencement of flowering.

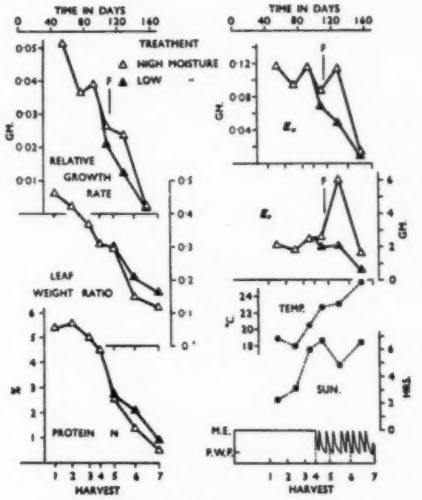


Fig. 2. Curves of relative growth rate, leaf weight ratio, leaf protein-nitrogen, and net assimilation rate on dry weight (E_W) and protein (E_P) bases. The climatic indices, mean maximum daily temperature and mean hours of bright sunshine per day for the appropriate inter-harvest periods are plotted above a soil-moisture diagram (see Fig. 1).

in the root systems of both linseed and flax, it would seem that the very much larger inflorescence of the former is able to initiate the breakdown and transport of carbohydrate and other reserves from the roots, and may indirectly arrest meristematic activity therein. The similar, though less pronounced decrease in stem weight after harvest 6 may be interpreted in the same way, since no such decrease was observed in the case of Liral Crown flax. Considerations such as these are in accord with the general principle that the distribution within the plant of carbohydrates and other metabolites is largely determined by the changing balance of demand set up by competitive meristematic tissues.[2] The basic pattern of this dynamic balance is an inherent characteristic

[1] See discussion of this point by Tiver (1942).

[2] This principle was successfully used by Petrie, Watson and Ward (1939) and by Watson and Petrie (1940) in their interpretation of the effect of topping in tobacco upon the subsequent ontogenesis of other organs.

of the species or variety under consideration, but the balance may be modified by many factors of the environment. One such factor is the water supply, and its modifying effect is well illustrated by the relative depression in yield of dry matter of different plant parts induced by the low moisture treatment of the present experiment. Thus at harvest 7 the leaves, stems, inflorescence and roots were depressed by 6, 27, 42 and 38 p.c. respectively. At the time of application of treatment, meristematic activity in the leaves had virtually ceased, that of the stems and roots was slackening, whereas that of the inflorescence had just commenced. During the period of treatment, therefore, the activity of the latter would tend to dominate all others and, as has been suggested above, might indirectly determine their continued activity.

Growth Analysis (Fig. 2).

In parallel experiments with two varieties of the same species it is to be expected that treatment effects and time trends in relative growth rate and its components will be similar.

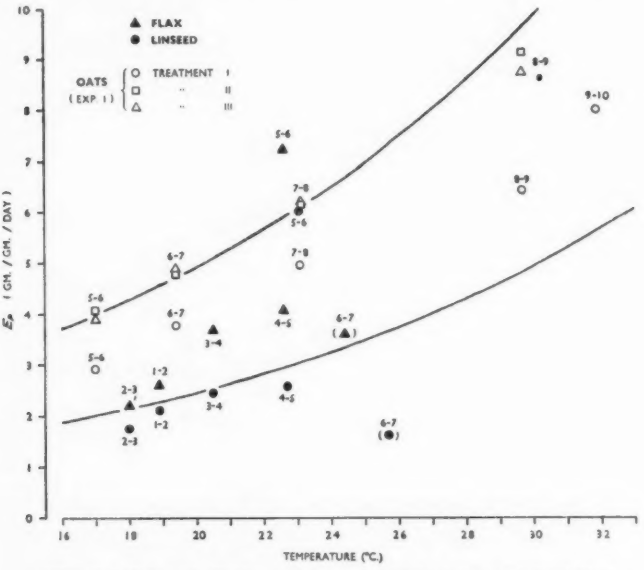


Fig. 3. Net assimilation rate on a protein basis (E_P) plotted against mean maximum daily temperature. The harvest interval is shown beside each point or pair of points, and the two values in parenthesis are for plants in an advanced stage of senescence. The data for flax are from Tiver (1942) and those for oats from Williams (1939). In the latter case the nutrient treatments were 0·0093, 0·121 and 0·486 gm. phosphorus per pot respectively. For further explanation see text.

This expectation is amply borne out by a comparison of Fig. 2 with the corresponding figure in the first paper of the series, and it follows that many of the observations made in that paper are equally applicable to linseed. Certain differences are, nevertheless, worthy of note. Thus the leaf weight ratios for linseed are consistently greater and the net assimilation rates, both E_W and E_P, tend to be at a lower level than those for flax. In linseed, too, the relative protein content of the leaves falls more rapidly than in flax after harvest 4, with the result that values are higher before and lower after flowering in the former variety. This difference is possibly associated with the larger inflorescence of linseed with its greater demand for nitrogen during development.

The low values of E_P for the first four harvest intervals together with the recurrence of a seemingly aberrant value for interval 5–6 call for detailed interpretation. To this end E_P values for flax, linseed, and for oats (Williams, 1939) are plotted against mean maximum daily temperature in Fig. 3. When growth was limited by the supply of nitrogen, Williams (loc. cit.) found

that E_P was determined primarily by temperature, and it may be shown from his data that the temperature coefficient, Q_{10} for E_P was 1·9 for oats (exp. 1) and 2·1 for Sudan grass. The two curves of Fig. 3 have therefore been calculated from the equation for Van't Hoff's rule assuming $Q_{10} = 2$ and with E_P rates at 17° C. of 2 and 4 gm. per gm. per day respectively. It will be seen that the upper curve is an approximate fit to the E_P values for the oat plants of treatments II and III, and Williams (1938) has shown that these high-phosphorus tested plants were limited in growth by their supply of nitrogen. Tentatively then, all values of E_P falling on this curve may be regarded as indicative of maximal efficiency of leaf protein when the latter is used as a measure of "the amount of growing material, so far as increase in dry weight is concerned" (Briggs, 1928, p. 285). Values of E_P falling on the lower curve would then be indicative of only 50 p.c. efficiency in this respect, and would derive from plants having a much higher nitrogen status. Assuming that temperature is the only limiting factor of the environment it is thus possible to use E_P as an inverse index of the nitrogen status of the experimental plants concerned. The E_P values for the low-phosphorus treated oats range in efficiency from 65 to 80 p.c. and thus indicate a relatively high nitrogen status which shows little change over a large portion of the life cycle of the plant.

The comparison of E_P values for flax, linseed and oats is rendered somewhat speculative by the absence of evidence as to the identity of the maximal efficiency of leaf proteins of different plant species and varieties. In the present case, however, the differences are so large that it seems reasonable to infer from the data for the first four harvest intervals that the nitrogen status of the flax plants was high and of the linseed very high. This conclusion gains support from the probability that the soil had a high nitrate-nitrogen content (see above). Furthermore, the difference between flax and linseed in this respect may arise from the fact that there were 60 flax plants but only 30 linseed plants per pot. For harvest-interval 5–6, however, the data suggest a sudden and considerable fall in the nitrogen status of the plants of both varieties. The explanation of this change may be sought in the absolute protein-nitrogen data for the leaves (Table 4), harvest interval 5–6 being the time of most rapid senescent breakdown of leaf proteins. Hanson (1941) found that surplus protein is produced in the chloroplast when the nitrogen supply is abundant and that, during net hydrolysis of leaf protein induced by drought, chloroplast-protein disappears more rapidly than does total protein. Rapid senescence of leaves of high nitrogen status would doubtless also result in the hydrolysis of surplus, and presumably non-functional chloroplast protein, thus automatically increasing the apparent efficiency of the remaining leaf protein. The above interpretation gains support from the data for wheat (Williams, 1939), where, in spite of an increase in temperature of only 2° C., the final E_P values for treatments III and IV (high nitrogen supplies) are each more than double their penultimate values. The final low values for flax and linseed (harvest interval 6–7) may be rejected because in advanced senescence carbon assimilation ceases to be a major determinant of changes in the total dry weight of the plant.

The interpretation here given for high E_P values during the phase of rapid hydrolysis of leaf proteins suggests that net assimilation rates expressed on a cytoplasmic-protein basis would be related more closely to environmental factors, and the effects of manurial treatments on E_P would tend to be eliminated. In this connection it is of interest to note that Wood (1941), using leaves of Sudan grass, found respiration rate to be highly correlated with cytoplasmic protein-nitrogen, but that the relation with total protein-nitrogen was more complex.

DISCUSSION.

An obvious difference between the flax and linseed plants studied in these experiments concerns their vegetative habit. The Liral Crown flax had tall slender stems with small basal branches whose growth was inhibited at an early stage. The Punjab linseed, on the other hand, had shorter stems and the two basal branches usually developed almost as well as the primary shoot. Dillman (1936) says that the characteristic habit of flax varieties is produced chiefly by means of a heavy rate of seeding (80 lb. or more per acre), but admits that the greater length, fineness, strength and yield of fibre in the fibre flaxes have been brought about by careful selection of superior types. Assuming 100 p.c. germination and survival, the rates of sowing in the present experiments were equivalent to 36 lb. per acre for linseed and 50 lb. per acre for flax. In a good season these rates would give satisfactory stands of winter-sown crops, and the densities of plants in the pots were of the same order as those found in well-grown crops in South Australia. The effect of these seeding rates on the nitrogen status of the two varieties was indicated

in the previous section of this paper, and it is conceivable that this difference would tend to favour the development of laterals in the linseed variety. It seems probable, however, that differences of spacing and nutrition merely modify the inherent characteristics of the varieties. Such characteristics may well be the expression of relative differences in the sizes of the apical meristems of the primary and secondary shoots.

Oil Yield of Punjab Linseed.

Data for seed and oil yield together with related quantities are set out in Table 3. The primary interest centres in the effects of the low moisture treatment, and these are best gauged by the relative indices in the right-hand column of the Table.

Dillman (1928) reports that severe drought dwarfed the vegetative growth of his plants (variety "Rio"), caused fully two-thirds of the flowers to blight without setting bolls, and reduced the number of seeds per boll. He says that drought apparently hastened the rate of oil deposition and the time of maturation of the seed, and his data suggest a slight decrease in oil content at maturity. Johnson (1932) found that delayed planting greatly reduced the yield of seed, both number of seeds per boll and number of bolls per plant being affected. Johnson also reported a slight reduction in oil content with delayed planting in one season, but no consistent differences in the next. In a survey of the effects of climate on the yield and oil content of four varieties of linseed grown at 54 field stations in North America, Dillman and Hopper (1943) conclude that both drought and excessive July temperatures decrease the yield per acre, the seed size, and the oil content of the seed. They suggest that such climatic conditions tend to shorten the growth period of the seed and force early ripening, thus limiting the period of oil synthesis. Field trials at this Institute (Waite Institute Report, 1941–2) have shown that a delay of only 18 days in sowing date reduced the seed yield of Punjab linseed from 15·7 to 5·4 bushels per acre; the oil content of the seed was also slightly decreased by late sowing. Percentage germination and the incidence of insect pests were of importance as determinants of yield in these trials.

In the present experiment the reduction in seed yield with low moisture treatment must be attributed to an observed reduction in the number of bolls per plant, and perhaps also to a reduction in the number of seeds per boll. The effect of treatment on the weight per 1,000 seeds is almost negligible. The inflorescence weight is depressed relatively more than is the seed weight by low moisture treatment, and it can be shown that the weight of the deseeded inflorescence with this treatment is only 48·6 p.c. of that of the control at harvest 8. This differential distribution of dry matter between the seeds and the inflorescence branches is yet another example of the effect of competition between different meristematic tissues; with low water treatment, the demand of the developing seeds for the limited supply of carbohydrates presumably tends to suppress the activity of the secondary cambium of the vascular tissues of the inflorescence branches.

The commercial value of the oil compared with that of the residual product, linseed meal, is such that even the small difference in oil content here recorded assumes significance. Apart, however, from deductions which may be made from agronomic data such as those of Dillman and Hopper (1943) little is known concerning the factors which determine oil content in linseed. The course of development of the oil in seed of linseed and flax varieties was studied by Dillman (1928) and Barker (1932) respectively. In seed from capsules of equal age the oil content increased rapidly during the second and third weeks after flowering, but thereafter there was no significant change up to full maturity (five to six weeks after flowering). Dillman also demonstrated that the increase in oil content was less rapid in

bulk samples from capsules of all ages. This might have been deduced from his first finding, but the fact that the final value for bulk samples was 2–3 p.c. lower than for early formed seed suggests that drought and late sowing may both reduce the oil content by an accentuated effect on late formed seed.

The data here presented are thus in general agreement with earlier work on the effects of drought and time of sowing on linseed, and the growth analysis provides a foundation for the physiological interpretation of such data. A fuller investigation of the factors governing oil content in linseed was beyond the scope of this experiment.

TABLE 3.

	Harvest.	Treatment. High moisture (H).	Treatment. Low moisture (L).	$\frac{L}{H} \times 100.$
Oven-dry weight of inflorescence (mg. per plant)	7	1,226·8	709·1	57·8
	8	1,229·3	710·5	57·8
Oven-dry weight of seed (mg. per plant)	7	700·9	463·4	66·1
	8	735·3	470·6	64·0
Weight per 1,000 seeds (gm.)	8	6·67	6·52	97·8
Oil content of air-dry seed (p.c.)	8	41·62	38·40	
		40·06*	38·66	
		42·74	39·78	
Mean		41·47	38·95	93·9
Oil yield (mg. per plant)	8	334·3	174·6	
		298·6	187·0	
		302·5	214·0	
Mean		311·8	191·9	61·5

* This seed sample was noticeably immature by comparison with the other two samples.

SUMMARY.

The present study of Punjab linseed (*Linum usitatissimum*, L.) concerns the physiology of growth and the production of oil in plants grown with an adequate supply of water, and, after flowering, in plants subjected to artificial drought. Comparisons are made with the data for Liral Crown flax presented in the first paper of the series.

The growth of the whole plant was depressed by low moisture treatment, and the growth analysis revealed that this was due to a depression of the net assimilation rate (protein basis). The oil yield at maturity was decreased nearly 40 p.c. by this treatment, and the oil content of the seed was slightly decreased.

Differences between flax and linseed with respect to the development of plant parts and the ratios of these to the weight of the whole plant are interpreted in terms of the general principle that the distribution within the plant of carbohydrates and other metabolites is largely determined by the changing balance of demand set up by competitive meristematic tissues. Punjab linseed has a very high inflorescence weight ratio, but the stem weight ratio is greatly reduced by comparison with Liral Crown flax.

Assuming temperature to be the limiting factor of the environment, net assimilation rate (protein basis) was used as an inverse index of the nitrogen status of the plant. Prior to flowering the nitrogen status of flax was shown to be high and that of linseed very high.

Attention is drawn to the similarity of the effects of drought and of late sowing in the field, and it is indicated that the data of this experiment are relevant to the interpretation of such field trials.

APPENDIX.

When these experiments were planned it was hoped that the production of fibre and oil might be linked up with certain aspects of carbohydrate and nitrogen metabolism in the respective varieties. This objective was only partially achieved, and certain analytical data which are too meagre to warrant full discussion elsewhere are presented in Tables 4 and 5. The protein-nitrogen data were used in the growth analyses, and in Fig. 2 of each paper. It will be noted that the protein nitrogen expressed as a percentage of the total nitrogen is consistently lower in the leaves of linseed than in those of flax. This tends to confirm the finding that the linseed plants had the higher nitrogen status.

TABLE 4.

Total- and protein-nitrogen contents of the leaves of flax and linseed.

Liral Crown flax.				Harvest.	Punjab linseed.			
Total-N. p.c.	Protein-N. p.c.	Total-N. p.c. Protein-N.	Absolute protein-N. mg.		Total-N. p.c.	Protein-N. p.c.	Total-N. p.c. Protein-N.	Absolute protein-N. mg.
6·65	5·47	82	1·98	1	6·78	5·38	79	2·39
6·10	5·23	86	4·09	2	6·70	5·57	83	6·66
5·38	4·66	87	5·10	3	5·84	5·00	86	11·07
4·78	4·12	86	5·69	4	5·26	4·49	85	13·96
3·42	2·86	84	4·18	5H	3·17	2·52	79	11·66
2·33	1·96	84	2·50	6H	1·82	1·38	76	5·81
1·26	0·85	67	0·85	7H	0·81	0·52	64	1·79
3·48	2·83	81	4·07	5L	3·49	2·67	77	11·52
3·03	2·23	74	3·04	6L	2·71	2·09	77	8·30
1·91	1·35	71	1·93	7L	1·40	0·91	65	2·98

H = high moisture. L = low moisture.

TABLE 5.

Water content and total sugars (reducing sugars plus sucrose) in the leaves and stems of Liral Crown flax.

	Water.		Sugars.	
Harvest.	Leaves. p.c.	Stems. p.c.	Leaves. p.c.	Stems. p.c.
1	758	775	0·63	2·46
2	769	729	0·89	2·86
3	720	579	1·07	4·21
4	648	445	1·66	4·60
5H	494	300	0·81	1·25
6H	396	222	2·00	2·96
7H	157	190	3·20	2·79
5L	389	255	2·77	1·67
6L	333	210	1·71	1·50
7L	91	176	2·63	2·44

H = high moisture. L = low moisture.

The total-sugar contents of the stems of flax were much greater than those of the leaves for all harvests prior to flowering, but thereafter they were of the same order in these plant parts. At harvest 5 the sugar content of the leaves was greatly increased and that of the stems slightly increased by low water supply. This seemingly anomalous effect is in accord with the findings

of Vasiljev (1931) and of Amos and Wood (1939) that, with decreasing water content of the tissues, the equilibrium between complex and simple carbohydrates shifts so that the concentration of the latter is greatly increased. The reversal in the treatment effects for harvests 6 and 7 might also be interpreted as equivalent to the fourth stage of desiccation described by Vasiljev, but the differences in experimental treatment are so great that it would be unwise to stress the similarity without more adequate data for flax.

Acknowledgments. The authors wish to record their indebtedness to the late Dr. A. H. K. Petrie for help during the planning of the experiment, and to Professor J. G. Wood for criticism of the manuscript.

REFERENCES.

Amos, G. L. and Wood, J. G. (1939): Austral. J. exp. Biol., 17, p. 285.
Barker, M. F. (1932): J. Soc. chem. Indust. Trans., 51, p. 218T.
Briggs, G. E. (1928): Proc. Roy. Soc., B, 102, p. 280.
Dillman, A. C. (1928): J. agr. Res., 37, p. 357.
Dillman, A. C. (1936): U.S.D.A. Yearbook of Agriculture, Washington, p. 745.
Dillman, A. C. and Hopper, T. H. (1943): U.S.D.A. Tech. Bull. 844.
Hanson, E. A. (1941): Austral. J. exp. Biol., 19, p. 157.
Johnson, I J. (1932): J. agr. Res., 45, p. 239.
Petrie, A. H. K. (1937): Austral. J. exp. Biol., 15, p. 385.
Petrie, A. H. K., Watson, R. and Ward, E. D. (1939): *Ibid.*, 17, p. 93.
Prescott, J. A. and Piper, G. R. (1930): J. agr. Sci., 20, p. 517.
Tiver, N. S. (1942): Austral. J. exp. Biol., 20, p. 149.
Vasiljev, I. (1931): Bull. appl. Bot., Genet., Plant Breeding, 27 (5), p. 47.
Waite Agricultural Research Institute, Report, 1941-1942 (in press).
Watson, R. and Petrie, A. H. K. (1940): Austral. J. exp. Biol., 18, p. 313.
Williams, R. F. (1938): *Ibid.*, 16, p. 65.
Williams, R. F. (1939): *Ibid.*, 17, p. 123.
Wood, J. G. (1941): *Ibid.*, 19, p. 313.

GERMINATING SEEDS AS A SOURCE OF VITAMIN C IN HUMAN NUTRITION

2. THE GERMINATING AND PROCESSING OF BLUE BOILER PEAS (*PISUM SATIVUM*) AS A PALATABLE SOURCE OF VITAMIN C

by J. W. H. LUGG AND R. A. WELLER

(From the Animal Nutrition Laboratory of the Division of Animal Health and Nutrition, Council for Scientific and Industrial Research, University of Adelaide).

(Accepted for publication 7th September, 1943.)

The preliminary work reported in a previous article (Lugg and Weller, 1943) had shown that germinated blue boiler peas deserved further investigation as a source of vitamin C in human diets. It remained to test the keeping qualities of the dormant seeds, to determine the methods of germinating the seeds in substantial quantities and under a variety of environmental conditions which would yield reasonably high concentrations of vitamin C, and to find, if possible, simple means of rendering the germinated peas palatable as food without sacrificing an unduly large fraction of the vitamin in the process. These aims were embraced in points (b), (d), (e), and (f) in the previous article (Lugg and Weller, 1943).

EXPERIMENTAL.

The ascorbic acid and "total" ascorbic acid (i.e. ascorbic acid plus dehydroascorbic acid calculated as ascorbic acid) contents of the materials were estimated by the methods described by Lugg (1942). No unusual difficulties were encountered and, as in the preliminary work, the estimations are believed to have been reasonably free from interference.

Three bulk lots of seeds (A, B and C) were used in the experiments. A, the lot used in the earlier work, was used in further experiments, and the age of the dormant seed over the entire period of work was 1·0–1·2 years. The age of B, a lot from the following harvest, was 0·1–0·3 years over the period. Lot C had been kept loosely covered in glass jars for 2 years at uncontrolled room temperatures (10° C.–45° C.).

Viability tests were made under the standard conditions described in the previous article (Lugg and Weller, 1943), by keeping the seeds thoroughly wetted on cotton wool at a temperature of about 22° C. (about 25° C. during the daytime and 20° C. at night) and exposed to diffused daylight at 3 ft. candles intensity for about 13 hours of each 24 hours. The "viability" was taken as that percentage of seeds which showed a protruding radicle within 96 hours.

The seeds were germinated under varying conditions. In two experiments made to test the effects of exposure to light, the light penetrated a sheet of glass which covered the peas. The under surface of the glass was either clear (transmitting 80 p.c. of the light) or frosted and partly blackened (transmitting 4 p.c.) or fully blackened (transmitting almost no light). The light used was sunlight, strongly diffused by passage through "frosted" glass, and frequently diffused and weakened in advance by cloud. The light intensities were measured with the aid of a photo-electric light meter.

Cooking trials were performed by dropping the germinated peas into water which had been heated to boiling in a vessel provided with a loose fitting lid, and raising to boiling again as rapidly as possible and keeping boiling for the remainder of the cooking period.

RESULTS AND DISCUSSION.

Keeping Qualities of the Seeds.

The excellent keeping qualities of these seeds were revealed by the viability tests on the three bulk lots (A, B and C). The conclusion was drawn that uninjured blue boiler peas have a viability of 99–100 p.c. at ages from 1 month to 2 years after harvesting. Germination was remarkably uniform. Almost every seed had germinated within 48 hours.

Variability of the Bulk Samples of Seed.

Five samples of A and one sample of B were tested for vitamin C production when germinated under conditions which were not strictly uniform in regard to illumination and temperature but approximated to the standard conditions imposed in the viability tests. The seeds were germinated

for 72 hours. The means of the results for A were: 0·28 mg. ascorbic acid and 0·36 mg. "total" ascorbic acid per gm. of tissue, the ranges of values being 0·24–0·32 mg. and 0·31–0·41 mg., respectively. The result for B was: 0·37 mg. "total" ascorbic acid per gm. of tissue.

There was nothing in these results to suggest that either bulk lot was superior to the other. The rather wide ranges of values for A were not anticipated as moderately uniform conditions obtained during the tests.

Effects of Exposure to Light.

Bulk lot B was used in this work. In the two experiments which were made the only departures from the standard conditions of germination were in regard to light intensity and temperature. The temperature was about 23° C. during the night and 28° C. during the daytime (12 hours), the mean temperature being 25° C.–26° C. As already stated the light source was diffused sunlight and the sky was frequently overcast. The seeds which received light were thus exposed for about 12 hours per day to light of widely varying intensity. During the first and last hour each day, extremely little light reached the peas, even when the sky was clear. In both experiments the germination period was 72 hours. Seedlings were harvested in the mornings after about 3 hours exposure to rather dull light. In the first experiment and during the first two days of the second, the light conditions were much the same each day, but in the second experiment the light was very dull during the last day.

The colour of most of the dry, dormant peas in the three bulk lots was pale grayish-green. Some 5 p.c. were pale fawn in colour and several per cent. were of mixed appearance.

Plumules of peas germinated in full light were green, in dim light greenish-yellow, and in darkness yellow. The cotyledons of the fawn-coloured peas were always yellow: those of the grayish-green peas germinated in strong light were yellow, in dim light greenish-yellow and in darkness green.

The vitamin C contents are shown in Table 1. It will be seen that exposure to light favoured the production of vitamin C. These findings appear to be in conflict with those of Virtanen and Ecrola (1936) who worked with peas (*Pisum sativum*) and found no difference between the ascorbic acid contents of seedlings grown in light and in darkness, but they appear to agree qualitatively with the findings of Reid (1938) for the chick pea (*Vigna sinensis*). Strict comparison is rendered impossible however, by the fact that these other workers estimated only ascorbic acid, not "total" ascorbic acid.

TABLE 1.

Production of vitamin C by blue boiler peas germinating under different conditions of illumination.

(Mean temperature, 25° C.–26° C. Period of germination, 72 hours.)

	Total amount of illumination (ft.-candle hours).	Stage of growth. Average length of a: ascending axes. d: descending axes.	Conc. of "total" ascorbic acid (mg. per gm. of tissue).
1st Experiment	< 15	a 1·0, d 3·5	0·34
	6,000	" "	0·52
2nd Experiment	< 15	a 1·0, d 3·5	0·32
	220	" "	0·36
	4,500	" "	0·44

Bogart and Hughes (1935) found no difference between the ascorbic acid content of oats germinated for 7 days in light and in darkness, and even after 13 days when pronounced differences between the pigmentations of the seedlings had developed, there was only a very slight and uncertain difference between the ascorbic acid contents.

Germination in Bags. Temperature Variation.

One of the most convenient methods of germinating large quantities of peas is to enclose them very loosely in a coarsely woven cloth bag or other type of perforated container which can then be immersed in water for any required periods of time, but almost all the peas of any considerable bulk so treated germinate in virtual darkness. Large scale germination in bright daylight requires that the peas be spread out in a thin layer and in such a way as to permit frequent or continual application of water, and this is not always convenient.

Various bag-germination tests were made. In all of them the bag and its contents were soaked in water for about 18 hours. Then the bag was suspended to drain. Thereafter it was immersed at intervals throughout the germination period and for about 1–2 minutes each time.

In two experiments peas from bulk lot B were germinated at a mean temperature of 25° C.–26° C. for 72 hours. One sample (1) germinated on wet cotton wool and at a light intensity of about 5 ft. candles for about 12 hours each day. The other sample (2) weighing about 0·5 kg. germinated in a small cloth bag which was immersed at intervals of about 3 hours

during most of the day but with an interval of about 13 hours each night. There was very little difference between the appearances of the two samples of peas after 72 hours or between the "total" ascorbic acid contents (0·37 mg. per gm. of tissue for (1) and 0·35 mg. per gm. for (2)).

In other experiments (3) and (4) respectively, 3·3 kg. and 11·5 kg. of peas enclosed in hessian bags were germinated for 72 hours under treatments corresponding very closely with that used in experiment (2). It was found, however, that the actual conditions of growth were very different from those obtaining in experiment (2). In experiments (3) and (4) the heat evolved during growth was not dissipated readily and the masses of peas rose considerably in temperature, especially during the night period of the final 24 hours, when the temperature rose from 26° C. to 35° C. The radicles, instead of being plump and about 3·5 cm. in length, were rather spindly and about 4·5 cm. in length. The "total" ascorbic acid contents were 0·25 mg. per gm. of tissue for (3) and 0·27 mg. per gm. for (4). For (4) the ascorbic acid content was 0·21 mg. per gm. of tissue. These contents are low compared with what could be expected of peas germinated in virtual darkness for 72 hours at 25° C.–26° C. The effect might be attributed to inadequate aeration or to loss of water which may have accompanied the rise in temperature. Cf. the observations of Ihuze (1940).

In two subsequent experiments, (5) and (6), 3·5 kg. lots of peas were germinated in hessian bags at 14·5° C. and 18·5° C. respectively, and maintenance of temperature and adequacy of aeration were assured by immersing the bags at intervals of 2 hours throughout the germination period following the initial 18 hours immersion. After 96 hours and 84 hours respectively, when the seedings closely resembled those germinated at 25° C.–26° C. for 72 hours the "total" ascorbic acid contents were 0·31 mg. per gm. of tissue for (5) and 0·29 mg. per gm. for (6). For (5) the ascorbic acid content was 0·25 mg. per gm. of tissue.

Cooking Trials.

The germinated peas were dropped into boiling dilute brine (3 gm. NaCl per litre) in the proportion of 1·0 kg. of seedlings per litre of brine. In the first trial the brine was made from distilled water, and in the second, from tap water of initial pH 8·0. Boiling had recommenced in 5 minutes and was continued for a further 15 minutes in the first trial, by which time the peas were tender, and for 30 minutes in the second trial, by which time the pea cotyledons were beginning to disintegrate.

Considerable water was lost by evaporation. After separating with the aid of a collander, the peas and the cooking water were weighed and sampled for the estimation of vitamin C. The moist drained peas were found to have increased in weight over the weight of the fresh material, by 9 p.c. in the first trial and 8·5 p.c. in the second.

The results of these trials are shown in Table 2. It will be seen that rather less than a third of the "total" ascorbic acid in the first trial and rather more than a third in the second trial were removed from the peas by the cooking, and that rather more than half of the amount was recoverable in the cooking water. The cooked peas were judged to be palatable by all six persons who tested them.

TABLE 2.

Effect of cooking upon vitamin C in germinated blue boiler peas.

		Raw peas.		Cooked peas.		Cooking liquid.	
		Conc.*	Quantity.†	Conc.*	Quantity.†	Conc.*	Quantity.†
1st Trial	Ascorbic acid	0·29	0·29	0·16	0·175	0·16	0·055
	"Total" ascorbic acid	0·39	0·39	0·25	0·27	0·18	0·06
2nd Trial	"Total" ascorbic acid	0·29	0·29	0·16	0·175	0·125	0·07

* Concentration in mg. of ascorbic acid or "total" ascorbic acid per gm. of material.

† Quantity in mg., of ascorbic acid or "total" ascorbic acid present in or derived from 1 gm. of fresh seedling.

CONCLUSION.

For a given quantity of air-dry, dormant, blue boiler peas germinated and cultured on water, the maximum vitamin C production would be attained by growing with access to bright light and probably until most of the bulk of the seedlings was represented by the axes. Such material could be eaten raw as a salad, but it would be rather tough and, it seems, distinctly lacking in palatability.

By germinating for much shorter periods, a less tough material is obtained and may be eaten raw as a salad, but the concensus of opinion is that it is far more palatable when cooked.

A usually satisfactory stage of growth is characterized by the descending axes being about 3 cm. in length and by the plumules just emerging. The seedlings contain 0·3 mg–0·5 mg. "total" ascorbic acid per gm., the lower values being obtained with growth in darkness. The loss of vitamin C by cooking in sufficient dilute brine to cover the peas (by boiling in a loosely covered vessel for about 20 minutes) is only about one-third. The cooking water, which contains about one-sixth of the total vitamin C, may be used for soups or stews. Thus grown in darkness, 60 gm. of air-dry seeds become about 137 gm. of seedlings, and when cooked as described weigh some 150 gm. and contain about 30 mg. of "total" ascorbic acid, and the cooking water contains about 8 mg. in addition. Incidentally, steam cooking, either in a properly designed "steamer" or by a modified boiling process with sufficient water to cover only the lowest layers of peas (e.g. Oser, Melnick and Oser, 1943), would almost certainly remove less vitamin C from the peas.

Although peas germinated for this shorter period will contain more vitamin C if grown in light, it is probable that the great convenience of germinating large quantities in bags or comparable containers, which is tantamount to germinating in virtual darkness, would outweigh other considerations.

The incidence of bacterial or mould damage during germination at temperatures of 15° C.–25° C. has been found to be very slight. Under conditions which would favour infection, some control could be exercised by harvesting at an earlier stage of growth and thereby sacrificing some of the vitamin C, or by employing permissible disinfectants. Bags and other containers should be sterilized frequently.

SUMMARY.

Good quality blue boiler peas have been found to retain their high viability (99–100 p.c.) when stored at room temperatures (10° C.–45° C.) for two years.

The peas have been tested for vitamin C production when germinated at temperatures of from about 15° C. to 25° C. and at varying intensities of illumination. Whilst the amount of vitamin C produced depends upon the stage of growth attained, but not significantly upon the temperature at which growth occurs, the variation with light intensity is rather marked. Thus when the descending axes are about 3 cm. long (after some 72 hours germination at about 25° C.) the seedlings contain from 0·3 mg. to 0·5 mg. of "total" ascorbic acid (i.e. ascorbic acid plus dehydroascorbic acid calculated as ascorbic acid) per gm. of tissue, depending upon whether growth has occurred in virtual darkness or in daylight.

Advantages and disadvantages of germinating large quantities of the seeds in cloth bags and similar containers have been discussed.

The seedlings are considered to be more palatable cooked than raw. When cooked by boiling for about 20 minutes in sufficient dilute brine to cover the peas, the loss of vitamin C from peas with descending axes about 3 cm. long is only about one-third, and about one-sixth is recoverable in the cooking water. Smaller losses could be anticipated in steam cooking.

We are indebted to the Catering Branch, Keswick Military Barracks, for valuable co-operation.

REFERENCES.

Bogart, R. and Hughes, J. S. (1935): J. Nutrition, 10, p. 157.
Ihuzi, Y. (1940): Zyozo Gk. Zassi, 18, p. 379.
Lugg, J. W. H. (1942): Austral. J. exp. Biol., 20, p. 273.
Lugg, J. W. H. and Weller, R. A. (1943): *Ibid.*, 21, p. 111.
Oser, B. L., Melnick, D. and Oser, M. (1943): Food Research, 8, p. 115.
Virtanen, A. I. and Eerola, L. V. (1936): Suomen Kemisilehti, 9B, p. 13.
Reid, M. E. (1938): American J. Bot., 25, p. 701.

SOME PHYSIOLOGICAL FUNCTIONS OF THE NUCLEUS IN AMOEBA, INVESTIGATED BY MICRURGICAL METHODS

by A. M. CLARK (Department of Zoology, University of Melbourne).

(Accepted for publication 3rd August, 1943.)

Due in greater part to the efforts of Chambers and Peterfi and their co-workers, micrurgy has been brought to such a stage of perfection that it is a most useful, and often indispensable, technique for the investigation of problems in cellular physiology. In an earlier paper (Clark, 1942) I gave a brief account of an investigation into the physiology of nuclear function in *Amoeba*, in which micrurgical methods were used. In this paper I propose to describe some experiments that support and add to the views already expressed.

Three problems will be considered. What are the effects of destroying the cell nucleus without actually removing it, and how are these effects modified by a multinucleate condition? What relations, if any, exist between the presence of the nucleus and the production of digestive enzymes, and do well-defined permeability differences exist between the different protoplasmic surfaces of an amoeba?

The apparatus used was the manipulator designed by Peterfi and manufactured by Carl Zeiss. The Peterfi electrically operated pipette was found to be inferior to the syringe injection system devised by Chambers, for it is not so readily controllable when injecting a small amount of liquid, while it breaks down completely when a pipette is used that has an orifice smaller than about 8μ. Most of the injections were made with the Chambers' injector, the small glass syringe being controlled by the fine adjustment of an old microscope stand. As the micrurgical technique has been well treated by the published papers of Chambers and Peterfi, no further details will be given in this paper.

The amoebae were from the stock used in the previous work. The usual wheat cultures were set up in Petri dishes and inoculated with *Chilomonas*. Pure lines were maintained throughout and each culture was subjected to routine examination once a week. In order to obtain multinucleate amoebae, rich cultures were allowed to stand for seven or eight weeks, when about 10 p.c. of the amoebae had two or more nuclei. Under normal culture conditions, less than 5 p.c. of the amoebae are multinucleate.

NUCLEAR INJURY IN UNI- AND BI-NUCLEATE AMOEBAE.

When the nucleus is removed from an amoeba, there is a 70 p.c. reduction in respiration, locomotion becomes very sluggish and infrequent, food is not digested and the amoeba dies after about seven days (Clark, 1942). As I was able to show that neither reduced oxygen consumption nor starvation were the direct causes of death, I suggested that cytoplasmic dedifferentiation might explain why enucleated amoebae always die.

In an attempt to find out whether this dedifferentiation was reversible or not, some experiments were made on transferring a nucleus from a normal amoeba into cytoplasm that had been enucleate for a few hours. As might have been expected, the experiment failed, for it did not prove possible to make the transfer without coagulating the nucleus. However, it did prove possible on two occasions to transfer a damaged nucleus from one amoeba to another nucleated individual, resulting in a binucleate amoeba, although one of the nuclei was coagulated and non-functional. In both cases, the supernumerary nucleus was digested.

In a second series of experiments, one of the nuclei of a binucleate amoeba was coagulated by pricking and subsequent agitation with a microneedle. The amoeba was then kept under continuous observation for several hours. After recovering from the shock of the manipulation, the amoeba regained its firm attachment and was very active for the whole period of observation. Five hours after the nucleus had been coagulated, it began to swell slightly and the peripheral chromatin granules began to disintegrate. Four hours later, the nucleus had broken up into

three small fragments and was obviously being digested. On the following day, only a few small fragments were left, the amoeba appearing as a normal, healthy mononucleate individual. Repetition of the experiment gave the same result, although there was a good deal of variation in the time taken for the damaged nucleus to disappear.

Pricking and coagulating the nucleus *in situ* in a mononucleate amoeba results in the production of an amoeba that behaves in the fashion characteristic of enucleate individuals. However, amoebae rendered functionally enucleate by this method only survive for two or three days, whereas those rendered enucleate by the actual removal of the nucleus live for seven days or more. Moreover, during the two days that an amoeba may survive after its nucleus has been pricked, there is no sign of digestion of the nucleus, which merely swells slightly but persists without any fragmentation occurring. Perhaps a certain amount of disintegration of the nucleus takes place, which, producing toxic substances, might explain why the amoebae die so soon. As will be demonstrated later, the failure on the part of the mononucleate amoeba to digest its own injured nucleus is due to the fact that cytoplasm with no nucleus at all cannot produce the necessary digestive enzymes.

On three occasions, amoebae with three nuclei were obtained from the cultures. It was possible to prick and coagulate two of the nuclei without causing the death of the amoeba, both nuclei being digested. Pricking all three nuclei resulted in the death of the amoeba with no digestion of the nuclei. The observations point very strongly to a relation between the production and functioning of digestive enzymes and the presence of an active nucleus.

I have noticed that bi- and tri-nucleate amoebae are always large specimens, indicating a possible correlation between the volume of nuclear material and the volume of cytoplasm. An interesting problem presenting itself is whether there is any decrease in cytoplasmic volume in a bi- or tri-nucleate amoeba that has had one or two nuclei destroyed *in situ* or removed. I have not yet made any experiments to investigate this point, but the results would be of value to the theory of nucleo-cytoplasmic ratios.

THE NUCLEUS AND DIGESTION.

The dependence of anabolic processes on the presence of a nucleus has been appreciated by all workers, while some authors have in addition maintained that digestion of food is also under nuclear control. The effects of destroying the nucleus by pricking support the latter view, as does the observation that the food vacuoles present in an amoeba persist after enucleation without undergoing any further changes. Food partially digested remains so. The following observations demonstrate very clearly that food digestion is definitely dependent upon the presence of a nucleus, in *Amoeba proteus* at least.

An amoeba with one nucleus was taken from the culture and placed in the moist chamber of the manipulator together with some small organisms as food. Within about ten minutes, the amoeba started to ingest a rotifer that was attached to the surface of the cover-slip. The process of ingestion was followed carefully until the food cup was completely formed. A small portion of cytoplasm containing the nucleus was then amputated with a microneedle, leaving the rotifer in a food vacuole in enucleate cytoplasm. Although not directly relevant to a consideration of digestion, an interesting observation may be mentioned here. Before the fragment of cytoplasm was cut off, it was noticed that the rotifer, by virtue of its active movements inside the food vacuole, was displacing some of the cytoplasmic granules in the neighbourhood of the vacuole. By watching the displacement of the granules, it was possible to form a mental impression of the viscosity of the cytoplasm. As soon as the amputation took place, the degree of displacement became appreciably greater, although, as far as could be judged, the rotifer was not showing any increase in its activity. In other words, immediately after enucleation, the cytoplasm showed a decrease in its viscosity. This may well be the result of the slight amount of mechanical stimulation that is unavoidable when drawing a thin needle through protoplasm.

The enucleated amoeba so produced lived for two days, the rotifer remaining alive and active in the food vacuoles for the whole period. When the amoeba died, the plasmalemma ruptured and allowed the food vacuole containing the rotifer to flow out into the external medium. In a few minutes, the rotifer had made its escape from the vacuole and moved away quite actively, but it died five hours later. Its death may have been due to inadequate oxygen supply during its sojourn inside the amoeba. However, the enucleate cytoplasm was quite unable to produce enough enzyme even to kill the rotifer, much less digest it. The experiment has been repeated and confirmed on three other occasions. A study of feeding in normal, nucleated amoebae showed that rotifers are usually killed within an hour, although some may live as long as two to three hours. In all cases, the digestive process is well advanced after 12 hours.

In contrast with this experiment stand the observations made by other workers besides myself, that enucleated amoebae frequently ingest small items of food, such as flagellates, which are killed but not completely digested. The discrepancy may be due to the fact that there is a storage of

digestive enzymes in the cytoplasm in quantities sufficient to kill small food organisms but not sufficient to kill a large organism such as a rotifer. It seems reasonable to suppose that a large rotifer would require a greater amount of digestive fluid for its treatment than would a small flagellate.

There would appear to be two possible explanations as to why an enucleated amoeba fails to digest food. Either the digestive enzymes are not produced by enucleate cytoplasm or else they are produced but, for some reason or other, are unable to exert their effects. It has already been mentioned that an enucleated amoeba can kill small organisms that have been ingested. This statement must now be qualified by adding that this is possible only in *recently enucleated* cytoplasm. An enucleate amoeba will ingest and kill food for the first two or three days of its survival, but after four or five days food is no longer ingested frequently, and even when it is, it lives for an exceptionally long period inside the food vacuole, as long as 24 hours, and then its death might well be due to its unusual environment and not to the action of a digestive enzyme. Now if there is sufficient storage of digestive substances to enable the enucleated amoeba to kill small food items during the first two or three days, why should not the food ingested at a later stage be killed? It cannot be due to the gradual utilization of enzymes, for if an amoeba is starved for three or four days to eliminate all food vacuoles, and is then enucleated and still kept under starvation conditions, food supplied and ingested on the sixth or seventh day after enucleation is not killed. In four of the five experiments made, it was necessary to inject the food, a specimen of *Chilomonas*, into the enucleated cytoplasm with a pipette, owing to the very slight degree of activity shown by an amoeba deprived of its nucleus for six days. It seems probable, then, that the failure of what I may be permitted to call an old enucleated amoeba to kill small ingested organisms may be due, not so much to a lack, or exhaustion, of digestive enzymes but to an inability of the enzymes present to function. The suggestion I make is that the enzymes concerned in digestion are formed in the nucleus up to the zymogen stage, and then diffuse through the nuclear membrane into the cytoplasm, where they become activated and carry out their digestive functions. The removal of the nucleus and the loss of the effects of gene action result in the gradual loss of the ultramicroscopic differentiation of the cytoplasm, leading to a failure on the part of the now dedifferentiated cytoplasm to activate the zymogen already present. It is necessary to assume a gradual and not sudden regressive change in the structure of the cytoplasm to explain the gradual loss of digestive power of the enucleated amoeba, unless we are to assume that the differentiating action of the genes persists for three or four days, and then suddenly stops.

PROTOPLASMIC SURFACE PERMEABILITIES.

Morita and Chambers (1929) drew attention to the differences in the permeability towards hydrogen ions shown by the nuclear and cytoplasmic surfaces of *A. dubia*. They found that whereas hydrochloric acid failed to pass through the plasmalemma, it passed readily from the cytoplasm into the nucleus and, provided the amount injected was not excessive, the acid had no toxic effects on the amoeba. Hence the free permeability of the nuclear membrane towards hydrochloric acid cannot be ascribed to damage to the nuclear membrane. This experiment has been extended by the use of twenty-two coloured substances soluble in water, in an endeavour to establish some correlation between the permabilities of the three main protoplasmic surfaces in an amoeba, the plasmalemma, the nuclear membrane, and the surface of the contractile vacuole.

No attempt was made to use solutions of equivalent strengths, as only qualitative differences in permeability were sought. In most cases, approximately 0·1 p.c. solutions in distilled water were used. Immersion of the amoeba in the coloured solution served to examine the permeability of the plasmalemma, while intracellular diffusion after microinjecting a small amount of solution

indicated the permeability features of the nuclear and contractile vacuole surfaces. Short descriptions of the injections made will be given only in the cases of five dyes that proved to be especially interesting.

Orthonitrophenol. The nitro and halo phenols are interesting because of the reversible action they have on cell division and cellular respiration. For example, 2: 4 dinitrophenol in small quantity increases respiration but blocks cell division, while in large quantities respiration is also blocked. The effects are reversible provided they are not prolonged (Bodine and Boell, 1938). Strong solutions of orthonitrophenol cause rapid cytolysis, but in concentration less than 1: 10,000, toxicity is low.

When immersed in a 1: 10,000 solution or when injected with a very small amount of the phenol, the amoeba shows a marked tendency to move in limax manner, although the single large pseudopod often forms two lateral bulges at the extremity, the amoeba appearing rather hammer-like. After prolonged immersion in very dilute solutions, 1: 15,000 to 1: 30,000, the amoeba becomes very sluggish, and shows the frequent streaming reversal and loss of attachment characteritic of an enucleated amoeba. (When using dilute solutions of orthonitrophenol, it is necessary to keep the preparations covered, as there is otherwise a gradual loss in strength of the solution. The phenol is surprisingly volatile).

After injecting a small amount of nitrophenol, there is marked contraction of the plasmagel tube at the posterior end, resulting in wrinkling of the plasmalemma and very rapid streaming of the plasmasol to form a single pseudopod. Attachment is lost for a few minutes only and the amoeba recovers completely and lives for several days after the injection is made. The phenol passes through the nuclear membrane but does not enter the contractile vacuole. If a large amount is injected, there is very rapid bulging of the plasmalemma followed by a slow redistribution of cytoplasmic granules, the reaction being typical of any large injection of a comparatively non-toxic substance dissolved in water. If the injection is big enough to rupture the plasmalemma, a few granules escape into the external medium, but there is always a rapid streaming towards the site of the rupture and the plasmalemma is repaired. The behaviour is then as if a moderate amount only had been injected. Before the rupture is repaired, it is possible to see the yellow nitrophenol passing out under pressure into the medium. As one of the effects of injecting nitrophenol is to increase the activity of the amoeba, the effects of an injection immediately after enucleation were examined, for enucleation always renders an amoeba very sluggish and frequently spherical and quite inactive. The nitrophenol was injected in small amounts at the same time as the nucleus was removed. There was no marked increase in activity, the amoeba slowly withdrew its pseudopodia and passed into the corrugated condition. Unlike the after-effect of an injection into a nucleated amoeba, no firm attachment appeared. In fact, within five minutes, the amoeba had lost its attachment and did not regain it for the two days that it survived.

It was repeatedly noticed that after injecting a moderate amount, very fine granules appeared in the clear areas beneath the plasmalemma bulges. This appears to be a reversible coagulation effect, for the granules disappear in a few minutes. They are very small, being close to the limits of microscopic visibility, but are very clear when observed with dark-field illumination.

Eosin. This dye is not very toxic and fails to pass through the plasmalemma. Injections were made with a half-saturated solution in distilled water. There is very rapid diffusion of the dye throughout the cytoplasm and nucleus, but it does not enter the contractile vacuole. The pink colour gradually fades in the cytoplasm, due either to a chemical reduction or to diffusion out through the plasmalemma, which would then be showing unidirectional impermeability. Even when the amoeba is destroyed by injecting a very large amount of eosin, the contractile vacuole remains impermeable and may drift uncoloured through the highly coloured medium for several seconds until it suddenly bursts like a bubble.

By injecting the dye into one large pseudopod near its tip, there is a gradual streaming towards that area and as soon as the nucleus enters the injected zone, it gradually becomes pink. The entrance of the dye into the nucleus is not due to damage to the nuclear membrane, for if only a small amount of dye is expelled from the pipette in such a way that it flows out on to the surface of the nucleus, a localized pink spot appears on the nuclear surface, and one can follow the gradual spreading of the pink colour to the opposite side of the nucleus. In about ten seconds, the dye can be seen to pass back into the cytoplasm, leaving the nucleus from over the entire surface of the membrane. The locomotion and behaviour of an amoeba injected in this careful manner are normal for hours afterwards. On the other hand, if after the nucleus has been stained a faint pink, it is agitated with a microneedle, the stain no longer passes back into the cytoplasm but is absorbed by the peripheral chromatin granules. This manipulation is followed by the death of the nucleus.

On account of its high colour index, eosin is easily detected in small amounts and it is possible to say that, in addition to failing to pass through the surface of the contractile vacuole, the dye did not enter any of the food vacuoles.

Acid fuchsin. Unless there is damage to the nucleus, there is complete reduction of the dye to a colourless leucobase, followed by very toxic effects. It was thus not possible to decide whether it passed through the protoplasmic surfaces or not.

Light red. This dye passes very slowly through the plasmalemma but passes readily into the nucleus, then diffusing back into the cytoplasm. Although the dye does not enter the contractile vacuole, it passes very slowly into the food vacuoles, which become stained a very bright red. A small injection suffices to stain all the vacuoles present. After forty-eight hours the food vacuoles, together with small segregation vacuoles, coalesce to form one large vacuole carried as a tail, and eventually extruded.

Methyl green. Passage through the plasmalemma is very slow and the dye is toxic. It does not enter the contractile vacuole but enters the nucleus rapidly, the food vacuoles more slowly. The nucleus is coagulated at once and the dye absorbed by the peripheral chromatin granules and, to a slight extent, by the karyosome. It is very surprising that in spite of its toxicity towards protoplasm, methyl green fails to enter the contractile vacuole. This was found to be the case with all the toxic substances used, and indicates the extraordinarily impermeable and resistant nature of the surface of the contractile vacuole.

The remaining substances used had no unusual effects and so to avoid a tedious description of routine injections, of which more than 150 were made, the results will be presented in tabular form.

TABLE 1.

Substance.	Plasmalemma.	Food vacuoles.	Nucleus.	Contractile vacuole.
Orthonitrophenol	+	+	+	—
Methylene blue	+	—	+	—
Neutral red	+	—	+	—
Methyl red	+	+	+	+
Alizarin S	+	+	+	—
Janus green	+	+	+	—
Acridine orange	+	+	+	—
Proflavine	+	+	+	—
Euflavine	+	+	+	—
Trypaflavine	+	+	+	—
Neutral violet	+	+	+	—
Methyl green	+	+	+	—
Methyl orange	—	—	+	—
Light red	—	—	+	—
Eosin	—	—	+	—
Azocarmine	—	—	+	—
Biebrich scarlet	—	—	+	—
Wool fast blue	—	—	+	—
Capri blue	—	—	+	—
Methyl violet	—	—	+	—
Phenosafranin	—	—	+	—
Magdala red	—	—	+	—

The significant facts that can be taken from the Table are: (1) All the substances passed through the nuclear membrane. (2) Only one entered into the contractile vacuole. (3) Those which passed through the plasmalemma also entered the food vacuoles. (Two exceptions). We can relate these differences to the normal functions of the different protoplasmic surfaces in the following manner.

When an amoeba is feeding, the food cups are formed directly from the plasmalemma, which necessitates a continual production of new plasmalemma while the amoeba is feeding. From this consideration, it is to be expected that the plasmalemma should have the same properties as the surface of a food vacuole. With the exception of the cases of methylene blue and neutral red, the two surfaces do show the same behaviour towards the substances injected. The plasmalemma, while maintaining its protective function in preventing the entry of deleterious substances into the internal protoplasm, must nevertheless allow certain types of molecule to pass through it, so that the amoeba can absorb any essential items from the medium, mineral salts for example. In the case of a protozoan such as a sporozoan, all food must be absorbed through the plasma membrane.

The food vacuole surfaces of an amoeba must allow the passage of digesting fluids and digested food. On the other hand, the nucleus, by allowing all types of molecules to pass through its surface membrane, is able to maintain a continuous

interchange of substances with the cytoplasm during interkinesis. Thus it is not necessary for the nucleus to enter mitosis, with the accompanying loss of the nuclear membrane, to allow substances formed in the nucleus by gene action to pass into the cytoplasm for activation. As certain kinds of highly specialized cells such as neurones do not show division, free permeability of the nuclear membrane towards a large variety of substances would be essential for the continued life of the cell.

By histochemical reactions, it can be demonstrated that a peroxidase occurs in the nucleus and cytoplasm of an amoeba (unpublished experiments). If the amoeba is starved, the reaction disappears from the cytoplasm, but reappears when feeding is again permitted, the enzyme tending to accumulate at the surface of the food vacuole. As the enzyme fails to appear in an enucleated amoeba that has ingested food, it is probable that the enzyme or a precursor is formed inside the nucleus and, passing out through the nuclear membrane, exerts its function at the surface of the vacuoles. The microinjection experiments described reveal that the permeability of the nuclear membrane is such that the enzyme could diffuse through quite readily, and are in agreement with the view that the enzyme is of nuclear origin. Recently Caspersson (1941) has suggested on the grounds of ultra-violet absorption spectrum analysis that the nucleus is the centre of protein synthesis, the protein diffusing through the nuclear membrane into the cytoplasm.

The apparently absolute impermeability of the surface of the contractile vacuole to all substances excepting water is in keeping with the view that this organ is osmoregulatory and not excretory in function. If the membrane were permeable to dissolved substances, at each systole there would be a loss of these substances which, under conditions in which the osmotic concentration of the external medium were low, would be a heavy drain on the amoeba's reserves.

SUMMARY.

In a multinucleate amoeba, only one of the nuclei actually present is necessary for the life of the cell, the others being supernumerary and inessential.

Digestive enzymes appear in the cytoplasm as a result of nuclear action.

It is suggested that the inability of an enucleated amoeba to digest food is due to the failure of zymogens to be activated in the dedifferentiating cytoplasm.

The nuclear membrane is freely permeable to dissolved substances. The surface of the contractile vacuole is permeable to water but impermeable to most other substances.

The permeabilities of plasmalemma and food vacuole surfaces are similar.

REFERENCES.

Bodine, J. H. and Boell, E. G. (1938): J. cell and comp. Physiol., 11, p. 41.
Caspersson, T. (1941): Naturwissenschaften, 29, p. 33. (Seen in abstract only).
Clark, A. M. (1942): Austral. J. exp. Biol., 20, p. 241.
Morita, Yun-ichi and Chambers, R. (1929): Biol. Bull. 56, p. 64.
Chambers, R. (1928): Chapter 2 in McClung's Microscopical Technique, Hoeber.
Peterfi, T.: Abderhalden's H. buch d. biol. Arbeitsmeth. Abt. V, Teil 2, 1, p. 479.

THE INFLUENCE OF METALS ON DIPHTHERIA INFECTION

by MARY H. PETHERICK[1] AND E. SINGER.

(From the Baker Medical Research Institute, Alfred Hospital, Melbourne).

(Accepted for publication 11th August, 1943.)

It is known that certain metals influence the production of diphtheria toxin. Pappenheimer and Johnson (1936) and Pappenheimer (1936) found that minute amounts of iron markedly influenced toxin production. The optimum amount of iron varied only between the extremely narrow limits of 0·0001 and 0·0004 mg. per ml. An increase in the iron concentration to 0·006 mg. per ml. almost entirely inhibited toxin production but did not interfere with the multiplication of the bacilli.

According to Mueller (1941) toxin production by *C. diphtheriae gravis* is unaffected by concentrations of iron which inhibit toxin production by *C. diphtheriae mitis* and *C. diphtheriae intermedius*. Some indications were obtained by Wohlfeil (1937, 1938) that iron and copper ions have a death-delaying action in the experimental diphtheria infection of guinea pigs.

The following investigations were designed to determine the influence of heavy metals on the intradermal diphtheria infection of guinea pigs.

MATERIAL AND METHODS.

Strain. One strain of *C. diphtheriae gravis* was used throughout these investigations.

Standardization of suspension. The growth from horse-serum agar slopes incubated for 16 hours was washed off with 5 ml. of tryptic broth, the bacilli were spun down and the deposit again washed with 5 ml. of broth. The washed bacilli were then suspended in tryptic broth and the turbidity of the suspension compared with standard $BaSO_4$ suspensions. Appropriate dilutions of the suspension were made in broth and the number of living bacilli determined by placing 0·039 ml. of the dilution delivered from a standard pipette onto a horse serum agar plate and evenly distributing this amount over 1/4 of the plate. The colonies were counted after 24 hours incubation.

The number of bacilli which, 48 hours after intradermal injection, caused infiltration and redness of 0·5 cm. diameter with central necrosis was designated as 1 unit.

The strain was recovered from the skin of the guinea-pigs after each experiment. These passages increased the virulence of the strain so that the number of bacilli constituting one unit decreased from 900,000 to 100,000. This variation in virulence did not affect the results of the investigations.

Toxin. Diphtheria toxin was supplied through the courtesy of Dr. Morgan, Director of the Commonwealth Serum Laboratories, Melbourne. The amount of toxin causing infiltration and redness 1 cm. in diameter 48 hours after intradermal injection was taken as 1 unit. For the batch of toxin employed the unit was 0·1 ml. of a 1/33,000 dilution. Tested against 1/250 A.U. the Lr and Lo doses of this toxin were 1/9,000 and 1/10,000 respectively.

Metal compounds. B.D.H. or Merck's analytical reagents were used with the following exceptions:

Ferri et ammonii citras B.P.

Hemin, kindly supplied by Mr. Rawlinson of the Walter and Eliza Hall Institute, Melbourne.

Crystalline guinea-pig haemoglobin prepared according to Aberhalden (1922).

Ferritine prepared according to Laufberger (1937).

Cytochrome c. was prepared according to Keilin and Hartree (1937).

Cytochrome oxidase was prepared according to Keilin and Hartree (1938).

The pH determinations throughout were made with the glass electrode.

Iron determinations. Wet ashing with HNO_3 and colorimetric determination according to Vogel (1939).

---

[1] Working under a full-time grant from the National Health and Medical Research Council.

In testing the action of a given compound a solution double the strength of that to be tested was mixed with an equal volume of a double strength suspension of bacilli or double strength dilution of toxin immediately before injecting. As the volume injected was always 0·1 ml. concentrations of solutions were standardized in respect of this volume.

In preliminary experiments the lowest dose of the different metals which affected the skin was determined. Half of this amount was used as the "tolerated dose" in the experiments.

In the following Table one experiment on the influence of copper sulphate and ferrous sulphate is given in detail:

TABLE 1.

No. of bacilli injected.	γ of metal injected.	Reaction in skin.
3,100,000 (5 units)	—	++
„	10 Cu"	±
„	1·5 Fe"	(±)
620,000 (1 unit)	—	+
„	10 Cu"	±
„	1·5 Fe"	(±)

++ Signifies the reaction usually obtained with 5 units of bacilli constituting a central necrosis of 1 cm. diameter surrounded by a strongly infiltrated area of 1·5 cm. in diameter with oedema.

\+ Reaction obtained with 1 unit of bacilli as described under "material and methods".

± Slight redness and infiltration without necrosis.

(±) Skin nearly normal; slight redness without infiltration.

The action of the compounds tested is summarized in Table 2. The effective dose of a compound was that which reduced the reaction obtained with 5 units of bacilli to a reading corresponding to either ± or (±) as described above.

TABLE 2.

Compound.	γ of metal. Tolerated.	Effective.
$FeSO_4$	50	1–2
Ferricitrate	34	8–17
Pot. ferrocyanide	>500	*500
Pot. ferricyanide	>500	*500
Hemin	>4·5	0·45
Ferritine	>50	2·5
Haemoglobin	>10	5·5
$ZnSO_4$	50	15
$CuSO_4$	25	10
$CoSO_4$	25	—
$NiSO_4$	50	—
$MnSO_4$	25	—

* Marked reduction of the reaction; no necrosis but reaction stronger than ±.

All the iron compounds tested influenced the reaction caused by the bacilli. The activity of the different compounds varied. The highest activity was found with ferrous sulphate, hemin, ferritine and haemoglobin. The chemical constitution of ferritine is discussed by Granick and Michaelis (1943). It is not known with certainty in what form iron occurs in ferritine. In the other compounds mentioned the iron is in the ionized state. The smaller activity of the ferric citrate, potassium ferrocyanide and potassium ferricyanide may be explained by the fact that the iron in these compounds is undissociated.

Of the other metals tested only copper and zinc showed a similar effect. Cobalt, nickel and manganese which are the three transition elements nearest iron in the periodical classification were inactive in tolerated doses.

It was noted that, whereas the bacilli could be recovered without difficulty from the site of injection when an iron compound was injected with the bacilli, it was sometimes difficult to cultivate bacilli from sites injected with bacilli and copper or zinc salts. The possibility had, therefore, to be considered that the action of iron was due to a different mechanism from that involved in the action of copper and zinc. As bacilli could always be recovered from sites injected with iron compounds, a bactericidal or bacteriostatic action of the iron compounds was out of the question, but an antibacterial effect of copper and zinc seemed likely. Therefore, the bacteriostatic action of all compounds employed in the animal experiments was tested on 10 p.c. horse serum agar slopes. The experiments were performed in the following manner. 0·039 ml. of a suspension of bacilli was placed on the surface of slopes which contained varying concentrations

of the compounds. The number of bacilli was adjusted so that 20–50 colonies developed on the control slopes.

TABLE 3.

Bacteriostatic action of compounds employed in vivo *tests.*

Compound.	γ metal in 0·1 ml. medium.	Growth.
$FeSO_4$	50	—
	30	—
	25	+
	20	+
Ferricitrate	34	+
	17	+
Pot. ferrocyanide	500	+
Pot. ferricyanide	500	+
Hemin	20	+
	15	+
Ferritine	50	+
$ZnSO_4$	50	—
	12·5	—
$CuSO_4$	25	—
	17·5	—
$CoSO_4$	60	—
	12·5	—
$NiSO_4$	50	—
$MnSO_4$	25	—

These *in vitro* experiments suggest that amounts of copper and zinc which inhibit the development of the skin reaction in guinea-pigs might exert a bacteriostatic action. The bacteriostatic concentration of the iron compounds, on the other hand, was very much higher than the effective concentration *in vivo*. It must be considered that only a fraction of the injected metal compounds remains in the skin, the larger part being removed within a few hours. To illustrate this point, an experiment in which the iron content of the skin following intradermal injection of ferrous sulphate was determined is given in Table 4.

TABLE 4.

Amount Fe injected γ	Time elapsed since injection.	Mass of skin (gm.).	Fe recovered γ.	Calculated normal amount of Fe in skin γ.	Amount of Fe remaining in skin γ.
50	1 hr.	0·562	40	7·5	32
25	1 hr.	0·302	20	4	16
50	4 hrs.	0·407	22·8	5·3	17
25	4 hrs.	0·532	15·7	7	8
—	—	0·619	8	8	—

According to some of the authors cited, iron compounds inhibit the formation of toxin and thus the reduction of the reaction caused by the intradermal injection of living toxin-free bacilli could be explained. In our opinion caution must be exercised in accepting this explanation as it would be remarkable if such minute amounts of metal could influence the metabolism of a virulent parasite in the tissues of a living animal. In the following experiments it could be shown that the action of metal compounds is not restricted to the inhibition of toxin formation, but that iron, copper and zinc interfere with the action of diphtheria toxin on the skin of the guinea-pig.

These experiments are particularly difficult to perform because of the high lability of ferrous salts, the ferrous ion being rapidly oxidized in dilute solutions. Control experiments with dilute sulphuric acid indicated that at a pH of 4·5 the diluted toxin was rapidly destroyed. The ferrous sulphate solutions used in the experiments set out in the following Table had a pH of approximately 5·4; this pH had no appreciable effect on the toxin solutions. Experiments in which the ferrous sulphate was dissolved in broth cannot give a true representation of the action of ferrous ions because the ion reacts with the constituents of the broth and is immediately precipitated. However, the results of experiments in which broth was the diluent agreed with those in which the ferrous sulphate was diluted with saline, therefore, we are entitled to conclude that the iron has an action on the toxin.

Only the compounds containing ionizable iron were active. The compounds containing non-ionizable iron and the zinc, cobalt, manganese, nickel and copper salts were inactive in this respect. Only these compounds were considered active which reduced the reaction caused by the intradermal injection of 1 unit of toxin to nil.

As already mentioned, the objection could be made that the acid reaction of the ferrous sulphate might cause a partial destruction of the toxin. This criticism could be overcome in two ways:

(a) by using an iron compound the solution of which has a neutral or slightly alkaline reaction, or

(b) by buffering the solutions with material which does not cause precipitation of the metal salts, as did the broth. Rabbit serum was used for this purpose.

Haemoglobin had been found to be only slightly less active than ferrous sulphate in controlling infection with living diphtheria bacilli, but appeared to have no action on the skin reaction to the intradermal injection of diphtheria toxin. Cytochrome c, however, was found to be markedly active. The results are set out in Table 5.

TABLE 5.

Results of animal experiments of activity of $FeSO_4$ haemoglobin-iron and cytochrome-iron on diphtheria toxin injected in alkaline saline solution, pH 7·3. Injected immediately after mixing.

Compound tested.	γ metal.	Reaction.
—	—	++
Haemoglobin	4·5	++
Cytochrome c.	6	0
$FeSO_4$	10	0

Results of animal experiments on activity on various metals on diphtheria toxin when injected in rabbit serum. Toxin and metals dissolved in saline and added to inactivated rabbit serum so that final concentration of serum is 50 p.c., pH 7·4. Injection immediately after mixing.

Compound tested.	γ metal.	Reaction.
—	—	++
$FeSO_4$	10	0
	2·5	0
$CuSO_4$	10	0
	2·5	0
$ZnSO_4$	10	+
	2·5	+
$MnSO_4$	10	++
	2·5	++

Strength of reaction: ++ one unit. + less than one unit. 0 no reaction.

An amount of cytochrome c containing 6γ of iron dissolved in saline at pH 7·4 and injected simultaneously with the toxin abolished the reaction that ordinarily ensues, whereas, a comparable amount of iron as haemoglobin was inactive. It is remarkable that haemoglobin is very active in controlling infection, and further, as shown in the second part of this paper, although it is nearly as active as cytochrome c in *in vitro* experiments, it is devoid of any activity when injected simultaneously with toxin. It seems likely that the haemoglobin molecule in the unaltered state is inactive, but that active iron compounds are liberated during incubation *in vitro* or some time after intradermal injection in guinea-pigs. Several other points have to be considered, particularly the fact that cytochrome-iron can be reversibly oxidized (can undergo a valency change), and further that the smaller molecular weight of cytochrome c could facilitate the interaction of the iron and toxin.

As one of us has shown (Petherick, 1942) the destruction of diphtheria toxin in the living animal occurs mainly in the organs, the blood with its high haemoglobin content being practically inactive. It is very likely, therefore, that cytochrome c and perhaps other iron containing cell constituents are important in the mechanism concerned.

Further, it was observed that ferrous sulphate was active in reducing the reaction which follows the intradermal injection of diphtheria toxin if it were injected together with the toxin in a solution containing 50 p.c. inactivated rabbit serum. The buffering action of the serum brought the pH of the mixture to pH 7.4. Zinc sulphate and copper sulphate were also active when injected intradermally with the toxin in the 50 p.c. serum solution. This activity had not been observed in the earlier experiments in which the solutions were made in broth or saline. From this varying activity in different solutions, it appears that a metal may be active only when present in a certain type of combination.

THE INFLUENCE OF METALS ON DIPHTHERIA TOXIN.

It is obvious that iron, copper, and zinc have a specific effect on diphtheria infection and intoxication. It is known that certain changes in the ascorbic acid content occur in the body of animals and man during diphtheria infections (Jungeblut and Zwemer, 1934–5). Biochemical studies (Edlbacher and Segesser, 1937; Edlbacher, 1937–8) have shown that ascorbic acid and iron can act together as a desaminating system. Further, it has been demonstrated by Maschmann (1938 a and b, 1939 a, b, c and d) and Naumann and Schinke (1941) that iron and other metals of the transition elements are essential constituents of different enzymes concerned with protein metabolism and that these enzymes are only active in the presence of redox substances. It seemed likely, therefore, that the metals might act in combination with ascorbic acid or other redox substances.

Experiments of two types were performed to test this hypothesis:

1. Dilutions of diphtheria toxin in Sörensen's phosphate buffer of varying pH were set up in test tubes and the appropriate additions of metal compounds and neutralized ascorbic acid made. The total volume was 3 ml., the toxin content per 0·1 ml. being 90 units. Samples were taken immediately after mixing and after 4½ hours at room temperature, and tested by injecting 0·1 ml. of a dilution containing 3 units of toxin, into guinea-pigs.

2. The above technique was employed, but a mixture of equal parts of saline and inactivated rabbit serum was used instead of the phosphate buffer.

The results of these experiments are given in Table 6. Slight variations in results occurred, hence every experiment is recorded, the figures indicating the number of tests made. The sign '++' represents the reaction usually obtained after injection of 3 units of toxin, '+' the reaction observed after injection of one unit, and '±' a reaction weaker than that of one unit. Thus in line 12, 1 mg. p.c. of ascorbic acid was added to the toxin. Immediately after mixing, in five different experiments, the intradermal reaction was '++'; this is recorded in the table as '5 ++'. After standing at room temperature for 4½ hours, 12 out of 15 single experiments gave '++' reactions, 3 gave '+' reactions. This result is given in the table as '12 ++,' '3 +.'

TABLE 6.

Results of experiments with sodium ascorbate.

(a) in M/30 phosphate buffer:
pH 6·9–7·1.

Line No.	Compound tested.	Metal mg. p.c.	Ascorbic acid mg. p.c.	Skin reaction. Before standing.	After standing.
1	—	—	–	6 ++	6 ++
2	$FeSO_4$	1·5	–	2 ++	1 ++, 1 +
3	„	0·6	–	1 ++	1 ++
4	Ferr. cit.	13·0	–	2 ++	1 ++
5	Haemoglobin	1·0	–	1 ++	1 ++
6	Cytochrome c.	1·0	–	1 ++	1 ++
7	$CuSO_4$	1·5	–	—	2 ++
8	$ZnSO_4$	1·5	–	—	1 ++, 1 +
9	$CoSO_4$	1·5	–	—	2 ++
10	$NiSO_4$	1·5	–	—	2 ++
11	$MnSO_4$	1·5	–	—	2 ++
12	—	—	1	5 ++	12 ++, 3 +
13	$FeSO_4$	1·5	1	1 ++	1 ++, 1 +
14	„	0·6	1	1 ++	2 +
15	„	0·3	1	—	2 +
16	„	0·15	1	—	2 +
17	Ferr. cit.	13	1	2 ++	1 ±, 2 0

TABLE 6 (continued).

Line No.	Compound tested.	Metal mg. p.c.	Ascorbic acid mg. p.c.	Skin reaction. Before standing.	After standing.
18	" "	6	1	—	1 0
19	" "	3	1	—	1 0
20	" "	1·5	1	—	1 0
21	Haemoglobin	1·0	1	1 ++	1 ++
22	"	0·5	1	—	1 ++
23	"	0·25	1	—	1 ++
24	Cytochrome c.	1·0	1	1 ++	1 +
25	"	0·5	1	—	1 ++
26	"	0·25	1	—	1 ++
27	$CuSO_4$	1·5	1	1 ++	2 0
28	"	0·6	1	—	1 0
29	$ZnSO_4$	1·5	1	1 ++	2 +
30	$CoSO_4$	1·5	1	1 ++	2 +
31	"	0·6	1	—	1 +
32	$NiSO_4$	1·5	1	1 ++	1 ++, 1 +
33	"	0·6	1	—	1 ++
34	$MnSO_4$	1·5	1	2 ++	2 ++
35	"	0·6	1	—	1 ++
pH 6·3–6·8.					
1	—	—	–	3 ++	3 ++
2	Ferr. cit	13	–	—	1 ++
3	Haemaglobin	1	–	—	1 ++
4	Cytochrome c.	1	–	—	1 ++
5	—	—	1	1 ++	2 ++
6	Ferr. cit.	13	1	1 ++	1 0
7	Haemoglobin	1	1	1 ++	1 +
8	Cytochrome c.	1	1	1 ++	1 +
pH 7·2–7·5.					
1	—	—	–	4 ++	4 ++
2	Ferr. cit.	13	–	1 ++	1 ++
3	Haemoglobin	2	–	1 ++	1 ++
4	Cytochrome c.	5	–	1 ++	1 ++
5	—	—	1	2 ++	2 ++
6	Ferr. cit.	13	1	1 ++	1 +
7	Haemoglobin	2	1	1 ++	1 +
8	Cytochrome c.	5	1	1 ++	1 +
(b) in inactivated rabbit serum:					
pH 7·6–8.					
1	—	—	–	4 ++	4 ++
2	Ferr. cit.	13	–	1 ++	1 ++, 1 +
3	$FeSO_4$	1·5	–	1 ++	1 ++
4	"	0·6	–	1 ++	1 ++
5	$CuSO_4$	1·5	–	—	1 ++
6	$ZnSO_4$	1·5	–	—	1 ++
7	—	—	1	3 ++	2 ++, 1 +
8	Ferr. cit.	13	1	1 ++	1 +
9	$FeSO_4$	1·5	1	1 ++	1 0
10	"	0·6	1	1 ++	1 +
11	$CuSO_4$	1·5	1	1 ++	1 +
12	"	0·6	1	1 ++	1 +
13	$ZnSO_4$	1·5	1	1 ++	1 +
14	"	0·6	1	1 ++	1 ++
15	$NiSO_4$	0·6	1	1 ++	1 ++

(a) *In M/30 Phosphate Buffer:*

(i) pH range 6·9–7·1. Here low concentrations of metal compounds are without effect on diphtheria toxin when incubated with toxin diluted with neutral phosphate buffer. A slight lessening of the toxin content was observed in two instances only, once when the toxin was incubated with 1·5 mg. p.c. ferrous iron and once with 1·5 m.g. p.c. zinc. Ascorbic acid in a concentration of 1 mg. p.c. does not diminish the toxin content.

If, however, the toxin is incubated with metal compounds plus ascorbic acid definite changes are observed. Some of the metals tested produced a marked detoxication. From the tests so far made it is rather difficult to assess differences in activity, nevertheless, it can be stated that in the concentrations employed copper and iron, the latter in the form of ferric ammonium citrate, are more active than iron as ferrous sulphate of cytochrome c, Zn++ and Co++, whereas Ni++, Mn++ and iron as haemoglobin are inactive.

(ii) pH range 6·3–6·8. The results obtained at this pH range were similar to those in the pH range 6·9–7·1. The toxin was found to be stable in this pH range and addition of ascorbic acid to such toxin solutions was without effect. In the presence of ascorbic acid, iron, as ferric ammonium citrate, was found to be very active in detoxifying the toxin. Haemoglobin and cytochrome c were slightly active.

(iii) pH range 7·2–7·5. In this pH range no marked difference between the activity of ferric ammonium citrate, haemoglobin and cytochrome c could be observed.

Thus it has been possible to demonstrate that the metals which were active in the *in vivo* experiments are also active *in vitro*. A metal compound which is able to suppress the reaction developing after the injection of diphtheria toxin when injected simultaneously with the toxin, is also able to detoxify diphtheria toxin *in vitro*. Much smaller amounts of metal are effective *in vitro* than would be necessary to demonstrate this effect *in vivo*.

(b) *In Inactivated Rabbit Serum.*

pH range 7·5–8. Slight changes in activity were observed. Ferrous iron is apparently more active than ferric ammonium citrate, copper or zinc in this medium. This corresponds closely to the action of these metals when injected with diphtheria toxin diluted in saline.

For a considerable time it has been shown that vitamin C is important in diphtheria intoxication. It was found, however, that in severe diphtheria intoxication the animal body is not depleted of vitamin C and, whereas diphtheria intoxication is more severe in scorbutic than in normal animals, the difference is not very marked. Hence, it was thought that other redox substances must be involved and these experiments were repeated substituting adrenalin and glutathione respectively for ascorbic acid. The results of these experiments are set out in Table 7.

TABLE 7.

Experiments with Adrenalin.

Line No.	Compound tested.	Metal mg. p.c.	Adrenalin mg. p.c.	Skin reaction. Before standing.	After standing.
(a) *In M/30 Phosphate Buffer pH 7.*					
1	—	—	–	2++	2++
2	—	—	–	2++	2++
3	$FeSO_4$	1·5	1	1++	1 0
4	Ferr. cit.	13·0	1	1++	1++
5	$CuSO_4$	1·5	1	2++	2 0
6	$ZnSO_4$	1·5	1	1++	1+
7	$MnSO_4$	1·5	1	1++	1+
(b) *In Inactivated Rabbit Serum pH 7·8.*					
1	—	—	–	1++	1++
2	—	—	1	1++	1++
3	Ferr. cit.	13·0	1	1++	1+
4	$FeSO_4$	1·5	1	1++	1+
5	$CuSO_4$	1·5	1	1++	1+
6	$ZnSO_4$	1·5	1	1++	1++
7	$MnSO_4$	1·5	1	1++	1++
Experiments with Glutathione.					
In Phosphate Buffer pH 7·0.					
1	—	—	–	1++	1++
2	—	—	1	1++	1++
3	$FeSO_4$	1·5	1	2++	2++
4	Ferr. cit.	13·0	1	2++	2±
5	$CuSO_4$	1·5	1	2++	1±, 1 0
6	$ZnSO_4$	1·5	1	1++	1++
7	$CoSO_4$	1·5	1	1++	1++
8	$NiSO_4$	1·5	1	—	1++
9	$MnSO_4$	1·5	1	—	1++

From these results it is apparent that the synergistic action with metal compounds is not characteristic of ascorbic acid. Other redox substances are also active.

Minor differences in activity were observed. Ferrous sulphate and zinc sulphate were inactive with glutathione. Manganese sulphate had some activity and ferric ammonium citrate was inactive with adrenalin in phosphate buffer but active in 50 p.c. inactivated rabbit serum-saline.

Too much emphasis must not be placed on these differences, as the number of tests performed was small. The possibility had to be considered that an increase in the activity of cytochrome might be demonstrable if cytochrome oxidase were present in the reaction mixture. A few experiments were performed in which diphtheria toxin was incubated together with cytochrome and cytochrome oxidase and ascorbic acid, but no difference in activity was observed. This might be due to the fact that the cytochrome oxidase preparation is a suspension not a solution and the tissue detritus present might protect the toxin against the action of the cytochrome-cytochrome oxidase system.

DISCUSSION.

It is definitely established that iron, copper and zinc compounds have a detoxifying action on diphtheria toxin. It has been possible to confirm *in vitro* the observation originally made *in vivo*. In the *in vitro* experiments it has been shown that metal compounds by themselves are inactive or only very slightly active, but their activity can be considerably increased by the addition of ascorbic acid, adrenalin, or glutathione. Comparable results were obtained in phosphate buffer and inactivated rabbit serum-saline. The detoxication occurred to more or less the same extent in buffered mixtures in the pH range 6·3–8·0.

No definite indication was obtained as to the mechanism of detoxication. The fact that heavy metals together with redox substances can detoxify the toxin, whereas singly they are inactive, makes it very probable that an oxidative process or an enzymatic process is involved, which is active only at a certain Eh.

An attempt was made to test this hypothesis in a series of manometric experiments. These experiments were performed in such a way that the oxygen uptake of toxin in the presence of iron or copper and ascorbic acid was measured by the Warburg method. No parallel between oxygen uptake and detoxication could be observed. The oxygen uptake of pulp and extracts of guinea pig liver with and without the addition of diphtheria toxin was measured by the same method to determine whether or not the toxin inhibits the respiratory mechanism, but no significant differences were observed. Parallel experiments in an oxygen atmosphere and an atmosphere of pure nitrogen could not be performed as war-time conditions made it impossible to obtain oxygen-free nitrogen.

The most likely oxidative process which would lead to detoxication would be an oxidative deamination. Attempts were made, therefore, to determine free ammonia, by the method of Parnas (1928) in samples of toxin incubated with ferrous sulphate and ascorbic acid. High ammonia values were found for unincubated toxin (1·25 ml. N/100 ammonia per ml. of toxin), and this value could not be decreased by dialysis of the toxin before performing the ammonia estimation. No increase in ammonia values was found in samples of toxin incubated with metal and ascorbic acid.

Deductions of a general nature which can be made from these experiments are:

1. Intradermally injected diphtheria toxin can be detoxified by the simultaneous injection of iron, copper or zinc compounds. Substances which do not occur in the animal body, for example, ferrous sulphate and cell constituents such as cytochrome c, show this activity.
2. In *in vitro* experiments it has been shown that the metals are only active in the presence of redox substances and, although direct proof is lacking, it therefore seems very probable that the detoxication is linked with an oxidation-reduction mechanism.

As the mechanism of detoxication has been partially elucidated it is permissible perhaps to discuss briefly the question of practical applications. The detoxication of diphtheria toxin *in vivo* by the application of metal compounds indicates that, given a suitable metal compound, antitoxic chemotherapy may be feasible. As a great mass of experience has been accumulated in the synthesis of chemotherapeutic compounds in which a heavy metal is the active constituent, preparation of a suitable iron, copper or zinc compound should not present insuperable difficulties.

SUMMARY.

The effect of certain heavy metal compounds on the skin reaction developing after intradermal injection of diphtheria bacilli or toxin has been investigated. It has been found that iron, copper and zinc compounds have a marked effect on the skin reaction developing after intradermal injection of living diphtheria bacilli.

Iron, copper and zinc compounds when injected simultaneously with diphtheria toxin in a solution containing 50 p.c. inactivated rabbit serum decrease the skin reaction, caused by the toxin, to a considerable extent. Cobalt, manganese and nickel salts are inactive in this respect.

Iron, copper and zinc compounds incubated with diphtheria toxin *in vitro* detoxify the toxin in the presence of ascorbic acid, glutathione or adrenalin.

Certain implications of these facts have been discussed.

REFERENCES.

Aberhalden, T. (1932): Physiologisches Practicum, 3rd Edition.
Baumann, E. and Schinke, O. (1941): Biochem. Z., 308, p. 130; Chemical Abstracts, 37, 11375, 2.
Edlbacher, S. (1937): Z. physiol. Chem., 250, p. 24.
Edlbacher, S. (1938): *Ibid.*, 254, p. 275.
Edlbacher, S. and von Segesser, A. (1937): Biochem. Z., 290, p. 370.
Granick, S. and Michaelis, L. (1943): J. biol. Chem., 147, p. 91.
Jungeblut, C. W. and Zwemer, R. L. (1934–5): Proc. Soc. exp. Biol., 32, p. 122.
Keilin, O. and Hartree, E. F. (1937): Proc. Roy. Soc., Series B, 122, p. 298.
Keilin, O. and Hartree, E. F. (1938): *Ibid.*, 125, p. 171.
Laufberger, U. L. (1937): Soc. Chim. Biol., 19, p. 1575.
Maschmann, E. (1938a): Naturwissensch, 26, p. 791.
Maschmann, E. (1938b): *Ibid.*, 26, p. 139.
Maschmann, E. (1939a): *Ibid.*, 27, p. 276.
Maschmann, E. (1939b): *Ibid.*, 27, p. 628.
Maschmann, E. (1939c): *Ibid.*, 27, p. 819.
Maschmann, E. (1939d): Biochem. Z., 300, p. 89.
Mueller, T. H. (1941): J. Immunol., 42, p. 353.
Pappenheimer, A. M., Jnr. (1936): Brit. J. exp. Path., 17, p. 342.
Pappenheimer, A. M., Jnr. and Johnson, S. (1936): *Ibid.*, 17, p. 335.
Parnas, J. K. (1928): Laboratoriums Methoden der Weiner Kliniken, Deutrike.
Petherick, M. H. (1942): Austral. J. exp. Biol., 20, p. 263.
Wohlfeil, T. (1937): Centr. Bakt., 1, pp. 139, 417.
Wohlfeil, T. and Becker, H. (1938): *Ibid.*, 142, p. 439.
Vogel, —. (1939): Quantitative Inorg. Anal., p. 701.

CHARACTERISTICS OF THE INFLUENZA VIRUS-ANTIBODY REACTION AS TESTED BY THE METHOD OF ALLANTOIC INOCULATION

by F. M. BURNET[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(Accepted for publication 13th September, 1943.)

Hirst (1942) has recently described a new method for the titration of influenza virus and for the estimation of antibody against the virus. In this method material is inoculated into the allantoic cavity and the presence or absence of infection determined by testing the fluid after two to three days incubation for the characteristic haemagglutinating action of the influenza viruses. A method essentially identical with that used by Hirst was independently developed in this laboratory and has been used extensively in the study of the antibody content of human sera (Burnet and Beveridge, 1943). The advantages of the method are (1) that it is immediately applicable to any influenza virus strain which will grow freely in the allantoic cavity, i.e., to all except completely unadapted "O" (Burnet and Bull, 1943) strains; (2) that the method is highly specific for the different A subtypes; and (3) that there is some evidence that the antibody titre measured in this way gives better evidence of resistance to influenza virus than titres deduced from other types of immunological procedure.

In order to provide a basis for the fuller interpretation of results with human immune serum a study has been made of the conditions under which neutralization occurs in the allantoic cavity. Interest has been particularly concentrated on the quantitative relationships of serum and virus at the neutral point.

TECHNIQUE.

The virus strain used for most experiments was BEL isolated in May, 1942. This strain is of type A (subtype "Melbourne") and was used in the form of allantoic fluid. Except where otherwise stated 4th passage material was used. Whenever a fresh stock was required a capillary tube containing 3rd passage virus was removed from a solid CO_2 refrigerator and 1: 100 dilution of this used to inoculate the required number of 10 day embryos in the allantoic cavity. After two days incubation at 35° C. the eggs were chilled and the allantoic fluid harvested free of blood. It was filtered through a sintered glass (G3) filter to remove cells and stored at 4° C. The other strains of virus mentioned in the text were also used in the form of allantoic fluid similarly collected and prepared.

The immune sera were from ferrets infected with the appropriate strains and bled two to three weeks later, or from human subjects experimentally immunized. All sera were inactivated at 56° C. for 30 minutes before being stored in the refrigerator.

For all types of neutralization test except the haemagglutination ones, serum and virus dilutions were prepared in ice-cold saline containing 10 p.c. of normal horse serum. Mixtures were left in the refrigerator for 1–2 hours before inoculation by the appropriate method. Standard methods were used for mouse and chorioallantoic inoculations and the technique of allantoic inoculation was that described by Burnet and Beveridge (1943).

For haemagglutination tests dilutions were made in Ca saline (0·04 p.c. $CaCl_2$ in 0·85 p.c. NaCl) since the presence of horse serum tended to give muddy agglutinations. Details of the method used are given in the paper by Burnet (1942).

The allantoic titration results were expressed as 50 p.c. end points computed according to Reed and Muench's method of accumulating positive and negative totals. Four to six eggs were used for each dilution or mixture. Fluid was taken from the eggs after three days incubation

[1] Work carried out with the aid of grants for research on virus diseases from the National Health and Medical Research Council and from Mr. E. Alec Cato.

for testing with fowl red cells. Since it is easy to remove a small portion of allantoic fluid without damaging the embryo repeated tests after two, three and four days incubation were made in several experiments to determine when the findings became stable. It was found that in at least two instances eggs inoculated with limiting dilutions or nearly neutral mixtures might be negative after two days but give a positive result on the third day. There were no instances in which any change occurred between third and fourth days.

EXPERIMENTAL RESULTS.

Effect of Time and Temperature.

The usual technique has been to allow mixtures to stand in the refrigerator for one hour before inoculation. The following experiment shows that the level of neutralization is little affected by modification of time and temperature within reasonable limits. Using the reagents BEL-allantoic-fluid-virus and homologous ferret immune-serum, F 179, a series of mixtures known from previous experiments to include the neutral point were inoculated (a) within a few seconds of mixing, (b) after 1 hour at 4° C., (c) after 1 hour at 35° C. The mixtures of b and c series were also diluted 1: 10 and 0·05 ml. of these dilutions inoculated after standing a further half hour at room temperature. Table 1 shows the results obtained.

TABLE 1.

Influence of time and temperature of contact on allantoic neutralization reaction.

	Serum-virus mixtures.											
	S + V.				S/3 + V.				S/10 + V.			
Immediate cold	−	−	−	−	+	+	+	+	+	+	+	+
1 hour 0°–4° C.	−	−	−	−	+	+	+	−	+	+	+	+
„ „ dil. 1: 10	+	+	+	−	+	+	+	+				
1 hour 35° C.	+	−	−	−	+	−	−	−	+	+	+	+
„ „ dil. 1: 10	+	+	−	−	+	+	+	−				

In this and similar tables S = undiluted serum, V = undiluted virus, + = embryo infected as judged by positive agglutination of fowl red cells by allantoic fluid, − = embryo not infected by same criterion, (+) or (−) = results obtained from dead embryos.

In another similar experiment summarized in Table 2 the same reagents were used but all mixtures were kept in the refrigerator, direct titrations and dilutions being made after 1 and 24 hours.

TABLE 2.

Time and dilution experiments made at ± 4° C.

	Original virus — serum mixtures.															
	S + V.				S/3 + V.				S/10 + V.				S/30 + V.			
1 hour	−	−	−	−	+	+	−	(−)	+	+	+	−	+	+	+	+
„ dil. 1: 10	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
24 hours	−	−	−	(−)	+	+	(+)	−	+	+	+	−	+	+	+	+
„ dil. 1: 10	+	+	−	−	+	+	+	−	+	+	+	(−)	+	+	+	+
„ dil. 1: 100	−	−	−	−	−	+	+	+	−	−	+	+	+	−		

These two sets of experiments show that at least with relatively concentrated reagents time of contact has little or no effect on the result either of direct inoculation or of inoculation after dilution. As a general rule mixtures were kept 1–2 hours in the refrigerator and would subsequently be left for a variable but short period at room temperature before inoculation.

Quantitative Relationships between Virus and Serum Concentrations at the Neutral Point.

It soon became evident that the ratio of virus to serum in neutral mixtures (i.e. producing 50 p.c. of infections) depended greatly on the absolute concentration of the reagents. If, for instance, undiluted virus mixed with an equal volume of undiluted serum gave a slightly over-neutralized mixture, virus diluted 1: 100 would not be inactivated by 1: 100 serum but would need a serum dilution of 1: 10 or less to reduce the proportion of infections to 50 p.c.

Using the reagents BEL (4th) virus and F 179 immune-serum a considerable number of experiments gave the data collected in Table 3 and shown graphically in Fig. 1. The virus used in different experiments was always of the same origin and gave titres (50 p.c. end-point) ranging between $10^{7·7}$ and $10^{7·0}$. A value of $10^{7·5}$ has been taken for the whole series. The Table

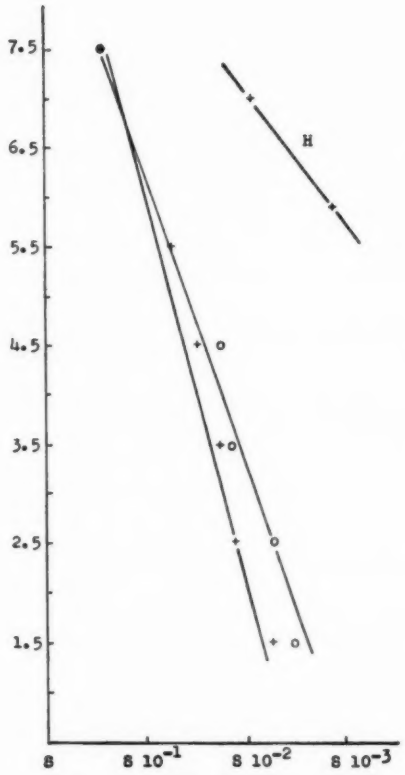


Fig. 1. *Neutralization curve for virus BEL 4th and serum F 179.*

Points marked + represent mixtures which under the standard experimental conditions would produce 50 p.c. of infections as judged from the data in Table 3. The straight line corresponds to the equation $\log V = 9{\cdot}7 + 3{\cdot}7 \log S$. Points marked o summarize the results obtained with mixtures allowed to stand 24 hours in the refrigerator before being tested in the allantoic cavity. Haemagglutination (Hirst) results are shown by line H.

Ordinates log concentration of virus in terms of 50 p.c. infective dose. Abscissae log concentration of serum in terms of undiluted material.

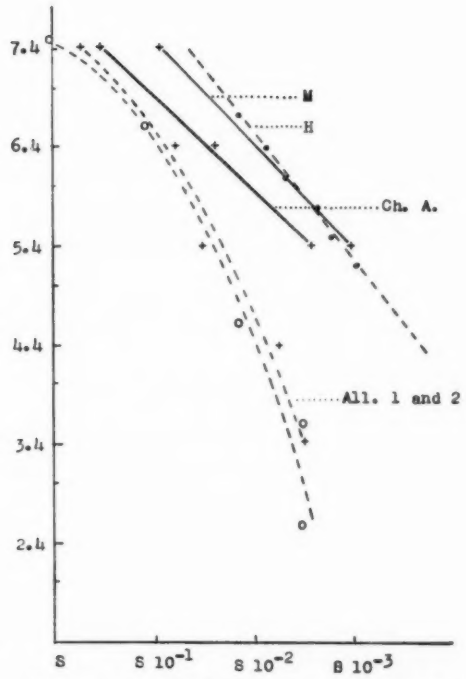


Fig. 2. *Neutralization curves for virus "W.S. Egg" and serum F 166 (anti-"W.S.") by four methods.*

Points marked × in the allantoic experiment and all results with the other three curves were obtained with a single batch of "W.S. Egg" allantoic fluid titre $10^{7{\cdot}4}$ and serum F 166. Points marked o were obtained in a second experiment with "W.S. Egg" virus of lower titre $10^{6{\cdot}6}$. Serum and virus concentrations shown logarithmically.

All. = allantoic, Ch. A. = chorioallantoic, M = mouse and H = haemagglutination methods of antibody titration.

shows for each dilution of virus the accumulated totals (Reed and Muench's method) from experiments with varying dilutions of serum. The final column shows the log. of the equivalent serum concentration.

TABLE 3.

Neutralization with varying concentrations of virus and immune serum.

Log virus concentration.	Log_{10} serum concentration. 0	− 0·5.	− 1·0.	− 1·5.	− 2·0.	− 2·5.	50 p.c. endpoint.
0	1—58	16—24	41—3				− 0·6
− 2			3—13	19—4			− 1·25
− 3			0—10	4—4	13—1		− 1·5
− 4				0—7	9—0		− 1·75
− 5				0—7	6—3	9—1	− 1·9
− 6				0—8	1—4	4—1	− 2·25

Each set of accumulated totals shows "eggs infected—eggs not infected".

From Fig. 1 it will be seen that when plotted logarithmically the points lie very close to a straight line with a slope considerably greater than 45°. The line corresponds to an equation of the type $x = by^a$, in the form $V = 10^{2\cdot2}\,S^{3\cdot7}$ or $\log V = 2\cdot2 + 3\cdot7 \log S$, where V and S are the concentrations of virus and serum in terms of the original undiluted reagents. When the virus concentration is expressed in 50 p.c. infective doses the equation for this example becomes $\log V = 9\cdot7 + 3\cdot7 \log S$. The constant b (in this instance 9·7) then represents the log of the amount of virus neutralized by an equal volume of undiluted serum. If two sera are to be compared against one virus and if both show the same value of a the relative activities of the sera are as shown in the equation $\log S_1 - \log S_2 = \frac{b_1 - b_2}{a}$. If for example a human immune serum used undiluted neutralizes virus BEL 3 (titre 7·5) at a dilution of 10^{-3} the value of b is 4·5. Compared with the standard ferret serum used in the experiments combined in Table 2 we have the relation $\log S_{F179} - \log S_H = \frac{9\cdot7 - 4\cdot5}{3\cdot7} = 1\cdot4$, i.e. the human serum corresponds in activity to a 1: 25 dilution of the immune ferret serum.

Although the results with strain BEL and F179 fall very close to a straight line in Fig. 1 there is a suggestion that the line may be a curve with a progressively steepening slope. More definite evidence to this effect was obtained with the strain "W.S. Egg" (adapted to the chorioallantois). This strain was chosen because it is the only available strain which can be fairly satisfactorily titrated by allantoic, chorioallantoic, mouse and haemagglutination methods. Neutralization tests by all these methods were made against the homologous ferret antiserum F166 and the combined results are shown in Fig. 2. In this Figure the points taken as representing "neutralization" for the different tests are, for allantoic tests 50 p.c. of infected embryos, for chorioallantoic titrations an average of 1 focus per membrane, for mouse tests average grade 2 consolidation involving about 1/5–1/6 of the superficial surface of the lung and for Hirst haemagglutination test the degree of partial agglutination which we call by convention "+". In order to make the results more directly comparable the end-point of neutralization for the mouse tests should theoretically be a considerably smaller degree of consolidation and the corresponding line in Fig. 3 should be moved about 0·3 unit to the left.

The lines corresponding to mouse, chorioallantoic and Hirst tests all have a slope close to 45° but both mouse and Hirst methods give lines somewhat steeper with values of a 1·1 and 1·27 respectively.

TABLE 4.

Neutralization tests with human immune serum.

Log. virus dilutions BEL 4th.	Log. serum concentration MAL (I.N.). 0.	− 0·5.	− 1.	− 1·5.	− 2.	Log serum concentration SHO (S.C.). 0.	− 0·5.	− 1.	− 1·5.	− 2.
0	0/4	4/1	5/0			0/4	0/5	4/0		
2		0/5	0/5	5/0			0/4	0/5	3/1	
4			0/5	1/4	5/0			0/5	1/4	3/2

The curve of allantoic neutralization begins almost parallel to the other curve but soon steepens and with virus diluted 1: 100 and more the slope is much the same as that found with BEL (4th).

With very dilute reagents prolongation of the time of contact before inoculation has a distinct effect. This is shown by the circles in Fig. 1. These represent results (not used in

obtaining the data for the line plotted) in which virus and serum mixtures had stood 24 hours before being inoculated. The best straight line to fit these points has a slope with a value of n 3·0 as against 3·7.

In order to determine whether the results with ferret immune sera are applicable to human antisera similar experiments were made with serum from two artificially immunized subjects who had shown an active antibody response. The subject MAL had been immunized intranasally with living attenuated virus while SHO had been given formalin-killed virus subcutaneously. (Table 4). When these results are plotted SHO gives a virtual straight line with $a = 3·7$ while MAL gives a curve very close to SHO for the lower two points but deviating to the left where the highest virus concentrations are being used.

Influence of Repeated Passage on Susceptibility to Neutralization by Serum.

There is a well marked difference in the readiness with which early passage and late passage substrains of the same influenza virus are neutralized by the same serum.

TABLE 5.

Comparison of neutralization of 1st and 60th egg passage strains of Melbourne (mouse).

Virus.	Log serum dilutions. 0.	— 0·5	— 1.	— 1·5	— 2.
M (1) undil.	0/4	0/10	6/0		
„ 10^{-2}		0/4	0/4	2/2	
„ 10^{-3}			0/4	1/3	4/0
„ 10^{-4}				1/3	3/1
	Titre 6·7.				

Virus.	Log serum dilutions. 0.	— 0·5	— 1.	— 1·5	— 2.
M (60) undil.	4/0	4/0			
„ 10^{-1}	0/4	4/0			
„ 10^{-2}		3/1	3/1		
„ 10^{-3}		0/4	4/0	4/0	
„ 10^{-4}			4/0	4/0	
		Titre 7·7.			

Table 5 shows the result of a comparative experiment in which a strain of "Melbourne" (M 60) which after adaptation to mice had had 55 amniotic and 4 or 5 allantoic passages was compared with the same strain (M 1) after only one allantoic passage. M 60 had a titre approximately 10 times that of M 1 but even making allowance for this it is much less readily neutralized by the homologous ferret serum used. M 1 at 1: 100 is equivalent to M 60 at 1: 10^3 but the serum concentrations required to neutralize these are respectively 1: 30 and 1: 5.

A similar comparison was made using the strain BEL after 42 egg passages in comparison with the standard 4th egg passage of the same strain. The same immune serum F179 was used throughout. Two consistent experiments with BEL 42nd gave the results used to determine the corresponding curve in Fig. 3. The constants for this curve are approximately $a = 4·0$, $b = 6·5$ in comparison with those for 4th passage BEL of $a = 3·7$, $b = 9·7$. The values of a are not significantly different so that the serum F179 has about 8 times greater activity against the 4th than against the 42nd passage ($\log 8 = \frac{9·7 - 6·5}{3·7}$). A similar difference between the two substrains was observed when they were tested against high titre human immune sera.

In this connection it is interesting to note the even greater difficulty with which the virus of Newcastle disease of fowls is neutralized in the allantoic cavity. This virus can be titrated by exactly the same technique but is of much greater virulence for the chick embryo than any of the influenza virus strains. The immune serum used was prepared by immunization of a rabbit with living virus (allantoic fluid) by the intravenous route. As judged by haemagglutination tests the serum was only slightly less active than an average influenza-immune ferret serum. Table 6 shows the results obtained in an experiment on allantoic neutralization.

TABLE 6.

Neutralization of Newcastle disease virus in allantoic cavity.

	Virus dilutions. 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Control							4/0	1/3
Serum R 6	6/0	6/0	4/2	1/5				
Serum R 6/10			6/0	6/0	6/0	6/0		

In Fig. 3 the three sets of results are plotted according to the convention used in Figs. 1 and 2. In all Figures the haemagglutination (Hirst) results are also shown. The distance separating the latter curve from that representing the allantoic results gives a measure of the resistance of the virus to inactivation by immune serum.

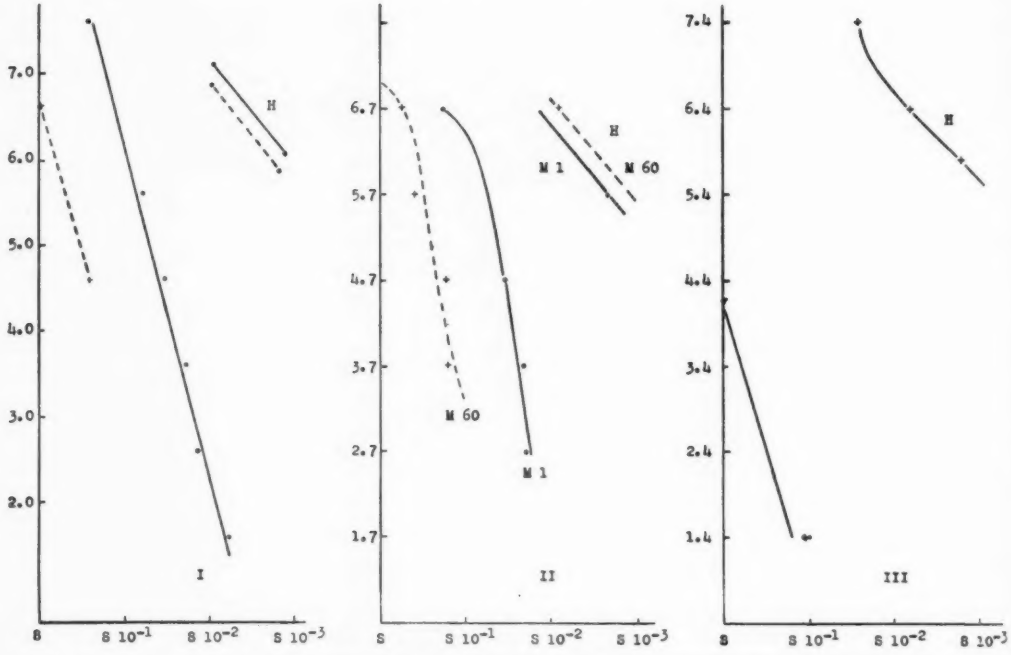


Fig. 3. *A comparison of neutralization curves of early and late allantoic passage strains of influenza virus and of Newcastle disease virus.*

In Section I strains BEL 4th passage (solid line) and 42nd passage (broken line) are compared; in Section II 1st (solid line) and 60th passage (broken line) of "Melbourne", and in Section III corresponding results with Newcastle disease virus are shown.

H in each section represents the points of neutralization as measured by haemagglutination (Hirst) test.

Dilution Experiments.

A number of experiments have been made in which slightly overneutralized mixtures of strong reagents were allowed to stand 1–2 hours in the cold and then diluted 1: 10 and 1: 100 before being tested. The dilutions were held from half to 1 hour before being inoculated. In all instances the serum dilution used in the primary mixtures was the smallest amount producing complete neutralization. In addition to F179 two high titre human sera MAL and SHO were tested against the virus strain BEL 3 and another experiment was carried out with "Melbourne 1" and the corresponding ferret serum as used in the experiment shown in Table 5.

TABLE 7.

Reactivation of inactive mixtures by dilution.

	V + S.	(V + S)/10.	V + S/100.
BEL 3 + F179	1—17	8—14	6—0
Melb. 1 + $\frac{\text{F.P.M.}}{3}$	0—6	0—6	0—6
BEL 3 + MAL	0—6	1—5	2—5
BEL 3 + SHO	0—6	0—6	2—5

Table 7 shows that a moderate degree of dissociation occurred in 3 of the 4 experiments but n comparison with direct experiments in which the same final dilutions were used shows that the dissociation was not complete.

DISCUSSION.

It is generally accepted that the virus-antibody reaction which results in inactivation is essentially similar to other antigen-antibody reactions. The inactivation results primarily from union of antibody to the virus surface. The union is at first reversible and most of the phenomena observed are best interpreted on the hypothesis of a dynamic equilibrium between virus, antibody and virus-antibody complexes (Burnet, Keogh and Lush, 1936). Union with antibody has no intrinsically destructive effect on the virus and the observed inactivation is a complex result of the interaction between susceptible cell and antibody-virus combinations. Further complications are introduced by the occurrence of aggregation and irreversible or less readily reversible types of union particularly with concentrated reagents.

The most interesting feature of the allantoic reaction as compared with mouse titration methods (Horsfall, 1939) or chorioallantoic membrane titrations (Burnet, Keogh and Lush, 1936) is the rapid loss of inactivating power as the immune serum is diluted. Instead of a value for the constant a in the formula $V = b\,S^a$ of 1 for chorioallantoic tests or 1·4 for mouse titrations, with the allantoic method the value lies between 3 and 4 if deviations in the zone of concentrated reagents are neglected. The difference can probably be related to the repeated opportunities for initiating infection that are available to a virus particle suspended in a large volume (5–8 c.c.) of nonviscous fluid and being constantly brought into contact with one or other part of an extensive sheet of highly susceptible cells. This differs sharply from the situation of a virus particle lodged in a thin film of fluid on the surface of the chorioallantois or on the respiratory epithelium of the mouse.

When virus is suspended in a solution of the homologous antibody the effect of the antibody is essentially to reduce the probability with which any given virus particle will initiate a lesion in the susceptible tissue, to some fraction of the probability in the absence of antibody. If the concentration of immune serum is such that this probability is reduced as in most of our experiments to a very small fraction, say $1/10^5$, we can picture any virus particle in the system as being united to a constantly changing number of antibody molecules. If it so happens that the number attached is low at the time the virus particle makes a suitable collision with the surface of a susceptible cell then infection will occur. On the other hand if the virus-antibody complex is in any way stabilized in the noninfective form, e.g. by adsorption to a cell surface, no infection can result. If we make the reasonable assumption that a virus uncoated or lightly coated with antibody is more readily adsorbed to a susceptible cell than one heavily loaded with antibody molecules then there will be a much greater proportion of multiple chances for a given virus particle to initiate infection in the allantoic cavity than in the more restricted arenas of other types of inoculation. Particularly with the lower antibody concentrations each virus particle will have on the average a longer period of indeterminate existence (i.e. before its fate is definitely decided) and therefore a longer period during which it could make an infective contact with a susceptible cell if the appropriate collision occurred. Such increased opportunity for infection would be reflected in an increasing steepness of the neutralization curve with dilution.

Another feature of considerable theoretical interest is the decreased susceptibility to inactivation of strains which have undergone prolonged allantoic or amniotic passage. In both the instances described above the change was of very considerable extent. An exactly similar finding was recorded in regard to neutralization tests made by the amniotic method (Burnet, 1941). This difference is not seen in haemagglutination tests, so that it provides rather direct evidence that an adapted strain requires a greater average loading of antibody molecules per virus particle to prevent infection of the allantoic epithelium than a strain not accustomed to this situation would require.

The steep slope characteristic of the serum-virus neutralization curves provides an adequate explanation for the wide range of titres observed in human sera. The fact that one serum can inactivate a million times as much virus as another does not imply that it contains a million times as much antibody. With the strain mostly used (BEL 4th) such a difference would correspond to a 30-fold difference in antibody content. Although there are insufficient data to allow a definite statement there seems to be no reason for assuming that essentially distinct antibodies are involved in any of the "neutralization" tests with influenza virus whether the indicator is the red cell, the allantoic cavity, the chorioallantois, or the mouse lung. The graphic representation of the four reactions in Fig. 2 suggests rather strongly that in each test the same type of antibody is concerned, the difference depending only on differences in the processes by which activity or otherwise of the virus is recognized.

SUMMARY.

A study has been made of the conditions which determine whether or not infection follows the inoculation of influenza virus-immune serum mixtures into the allantoic cavity of 11-day chick embryos.

When the concentrations of virus and immune serum giving neutral (50 p.c. infection) mixtures are plotted logarithmically they fall over most of the range on an approximate straight line corresponding to the equation $V = b\,S^a$ where a has a value of 3–4, i.e. the neutralizing power of the serum falls off rapidly on dilution.

In most experiments there was a significant deviation from a straight line when the reagents were used at high concentration. Here the slope corresponded to a value of a nearer to 1.

By choosing suitable mixtures well marked reactivation by dilution could be demonstrated; the diluted mixtures were however less active than mixtures of the same final constitution made by combining initially diluted reagents.

Influenza virus strains well adapted to growth in the allantoic cavity are more difficult to inactivate by serum than strains with only a few passages in this situation.

A comparison by several methods of the inactivation of the strain "W.S. Egg" by homologous immune ferret serum shows that the Hirst haemagglutination method shows the greatest apparent activity of the serum and the allantoic method the least. Mouse-lung titrations and titrations by the chorioallantoic pock counting methods gave intermediate values.

The bearing of these results on the titration of human immune sera is discussed.

REFERENCES.

Burnet, F. M. (1941): Austral. J. exp. Biol., 19, p. 39.
Burnet, F. M. (1942): *Ibid.*, 20, p. 81.
Burnet, F. M. and Beveridge, W. I. B. (1943): *Ibid.*, 21, p. 71.
Burnet, F. M. and Bull, D. R. (1943): *Ibid.*, 21, p. 55.
Burnet, F. M., Keogh, E. V. and Lush, D. (1937): *Ibid.*, 15, p. 231 (Supplement).
Hirst, G. K. (1942): J. Immunol., 45, p. 285.
Horsfall, F. L. (1939): J. exp. Med., 70, p. 209.

TRANSFORMATION OF VERDOHAEMOCHROMOGENS INTO MONOAZA-HAEMINS

by R. LEMBERG[1]

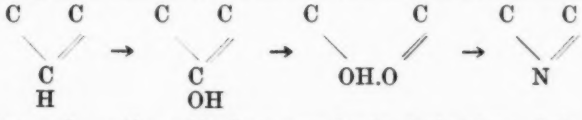
(From the Institute of Medical Research, Royal North Shore Hospital, Sydney).

(Accepted for publication 4th September, 1943.)

Aza-porphyrins are porphyrins in which one or several of the methene groups (CH) linking the pyrrolic rings in the porphyrin system are replaced by tertiary nitrogen (N). They are interesting intermediates between the porphyrins and the synthetic phthalocyanines of Linstead. According to the systematic nomenclature introduced by Helberger (1937) the latter are tetrabenzo-tetraaza-porphins. Azaporphyrins were prepared synthetically by Fischer and his collaborators and called imido-porphyrins (Fischer *et al.*, 1935; Fischer and Friedrich, 1936; Metzger and Fischer, 1936; Fischer and Müller, 1937; Fischer and Endermann, 1937; Endermann and Fischer, 1939).

Monoaza-porphyrins were obtained by heating αα-dibromo-dipyrromethenes with sodium hydroxide (Fischer and Friedrich, 1936), as by-products of diaza-porphyrins by heating the dibromo-dipyrromethenes with ammonia (Fischer and Müller, 1937), or from dipyrromethenes containing a urethane group in one and a methyl group in the other α-position (Endermann and Fischer, 1939). None of these reactions is well defined, their mechanism is complicated by partial breakdown of the dipyrromethenes yielding building stones for the synthesis of the monoaza-porphyrins, the conditions are rough and the yield poor. The aza-porphyrins form complex iron salts which closely resemble haematin compounds derived from porphyrins and which are very stable to acids.

In this paper a method is described by which monoaza-haemins are formed from verdohaemochromogens under much milder conditions. The verdohaeochromogens are green haemochromogens which are obtained from the red haemochromogens with porphyrin nucleus by the action of oxygen in the presence of reducing substances, e.g. hydrazine hydrate (Warburg and Negelein, 1930; Lemberg, 1935) or ascorbic acid (Lemberg *et al.*, 1938a). They are intermediates between haematins and bile pigments, closely related to the biliverdins into which they are readily transformed with loss of iron. Their formation is preceded by the introduction of an hydroxyl group on that carbon atom which is then eliminated with oxidative scission of the porphyrin ring. This initial reaction leads to the formation of haemochromogens derived from hydroxyporphyrins (Lemberg *et al.*, 1938 b), which then are further oxidized to verdohaemochromogens. The series of reactions leading from haemin to aza-haemin can thus be written in shorthand:



The primary formation of hydroxyporphyrin derivatives, their oxidation to verdohaem compounds and the transformation of the latter to biliverdins have been confirmed by Fischer and his school (Fischer and Libowitzky, 1938; Libowitzky and Fischer, 1938; Libowitzky, 1940; Stier, 1942). These authors believe, however, that verdohaemochromogens still contain a closed ring system, which is opened only during their treatment with acids. They also consider the verdohaemochromogens as ferric compounds (parahaematins), not as ferrous compounds (haemochromogens).

[1] This research was carried out with a grant of the National Health and Medical Research Council of Australia.

NOTE ON NOMENCLATURE.

Scientists not familiar with the field of haemoglobin and bile pigment chemistry must understand that the difficulties of nomenclature are to a large extent due not to the inability of the authors to come to terms, but to intrinsic difficulties caused by the large number of variables (nature and order of the side chains, nature and valency of the metal in complex combination, nature of addition compounds, nitrogenous and non-nitrogenous), to which is now added the new variable of altered tetrapyrrolic nucleus. A systematic nomenclature, e.g. that invented by Fischer for bile pigments, becomes so unwieldy that it is not used. The nomenclature of Clark and Drabkin suggested for metalloporphyrins (cf. Annual Rev. of Biochem. 11, 542 (1942)) is useful and is used in Table 1 below, but it suffers also from defects. It does not allow short summarizing terms as "haemins", "haemochromogens" or "parahaematins" and these are therefore still used in this paper. It is also not readily extended into the field of bile pigment haematin compounds, in which the metal-free compound as such is not known.

There is a tendency to oversimplify matters by the objection to the introduction of new names. An example is the extension by German and American authors of the term verdohaemochromogen, given by me to a definite and well characterized type of compounds, to cover all bile pigment haematin compounds. This has led to the confusion that we have now two types of "verdohaemachromogens" and two "verdoglobins" with widely different absorption spectra. While it is correct that identification of two compounds by spectroscopic means has to be accepted with reserve, surely two compounds with widely different absorption spectra cannot be identical and should not receive the same name.

EXPERIMENTAL.

Preparation of Pyridine Verdohaemochromogen and Pyridine Verdomeso-haemochromogen.

Pyridine verdohaemochromogen was prepared from crystalline haemin in the way described by Lemberg (1935) with the following modifications. A slightly higher reaction temperature (60° C. in the reaction mixture, 70° C in the water bath) was used. About one minute after the beginning of oxygenation the reaction mixture turned green. Reduction of a sample with sodium hyposulphite showed that at this stage little verdohaemochromogen was present; the solution contained the green parahaematin of hydroxyporphyrin, which by hyposulphite is reduced to its red haemochromogen.[2] The oxygenation was continued until a sample pipetted into a test tube containing some hyposulphite remained green and no trace of the absorption band at 557 mμ could any longer be observed with the spectroscope. The reaction was usually complete in five minutes and was then stopped at once by turning off the oxygen current and cooling with ice thrown into the solution. The yields were somewhat smaller than with the earlier method, but the product was entirely free from protohaematin and hydroxyporphyrin-haematin compounds. It was worked up in the manner previously described. The final precipitate obtained by adding petrol ether to the pyridine solution was redissolved in a few ml. of dry chloroform. The solution was filtered and without delay reprecipitated with petrol ether. In this way chloroform-insoluble products which had been formed during the isolation were removed.

Verdomesohaemochromogen was prepared from crystalline mesohaemin in the same way.

Preparation of Monoaza-Haemin from Verdohaemochromogen.

In 1935 I had already observed a profound alteration of the absorption spectrum of verdohaemochromogen by ammonia with the appearance of a single band in the green part of the spectrum. Other alkalis failed to produce this phenomenon. If freshly prepared verdohaemochromogen is dissolved in 10 p.c. ammonia and left standing at room temperature, the solution becomes brown and later purple. The three typical absorption bands of verdohaemochromogen at 655, 535 and 501 mμ disappear and are replaced by an absorption band at 566 mμ. On reduction with hyposulphite the band is greatly increased in strength and shifted to 572 mμ. As will be seen below, the compound with the band at 566 mμ is ammonia ferri-monoaza-porphyrin (parahaematin), which by hyposulphite is reduced to the ferrous compound (haemochromogen).

The reaction can be carried out in the absence of atmospheric oxygen. A few mg. of verdohaemochromogen are placed into the bent stopper of a Thunberg tube which contains a few ml. of 10 p.c. ammonia. The tube is repeatedly evacuated and filled with oxygen-free nitrogen. After mixing, the solution soon becomes purple and a strong absorption band at 572 mμ becomes noticeable. The fact that in the absence of oxygen the ferrous form is obtained directly, indicates that verdohaemochromogen is a ferrous compound.

300 mg. of verdohaemochromogen are dissolved in 50 ml. of conc. ammonia. After standing for one hour at room temperature the solution is refluxed for 30 minutes. It is then acidified with 100 ml. of glacial acetic acid and allowed to stand for 15 minutes. The brown-red aza-haematin is extracted by shaking repeatedly with ether and a dark precipitate is removed. The ether

[2] Drabkin (Annual Rev. of Biochem. 11, 539 (1942)) has overlooked that the colour and absorption spectra of these compounds have been described in a previous paper (Lemberg *et al.*, 1938b).

extract (total volume 1 l.) is washed several times with water, and then repeatedly shaken with small volumes of 20 p.c. hydrochloric acid. This removes purple bile pigments, which have been formed by the action of acetic acid on bile pigment haemochromogens derived from verdohaemochromogen by oxidation, and transforms aza-haematin into aza-haemin. The later is stable to acid and is not split, but a small part of it is lost by extraction with the strong hydrochloric acid. The ether solution is washed once more with 2 p.c. hydrochloric acid, concentrated to 30 ml. by distillation and filtered through a dry filter. On standing monoaza-haemin crystallizes in short straight needles. The filtrate is once more washed with water to remove acetic acid, shaken with 2 p.c. hydrochloric acid and concentrated. A second crop of crystals is obtained. Total yield: 65 mg. The mother liquor still contains monoaza-haemin.

Attempts to recrystallize from pyridine-acetic acid in the presence of sodium chloride or hydrochloric acid failed. On addition of ether incompletely crystalline material was obtained. Finally the crystals were dissolved in a small amount of hot glacial acetic acid; this solution was poured into a large volume of ether, filtered at once, shaken with 2 p.c. hydrochloric acid and worked up in the way described above.

Analyses (carried out by Dr. G. Burger at the Department of Chemistry of Adelaide University):

	Found.	Calc. for $C_{33}H_{31}O_4N_5FeCl$.	For $C_{33}H_{35}O_6N_5FeCl$.
C	58·0	60·68	57·50
H	4·80	4·79	5·12
N	9·80	10·73	10·17

The analyses leave no doubt that one additional atom of nitrogen has entered the molecule. The carbon and nitrogen contents are found somewhat too low and correspond more to those expected for a monoaza-haemato-haemin than for monoaza-protohaemin. The difference of the absorption maxima of various derivatives from those of the corresponding meso derivatives (cf. Table 1) are, however, those to be expected for the proto compound.

Cholehaemochromogen could not be transformed into monoaza-haemochromogen by ammonia. This confirms the view that cholehaem is different from verdohaem.

Monoaza-Mesohaemin from Verdomesohaemochromogen.

The preparation was exactly parallel to that of monoaza-haemin except that verdomesohaemochromogen was used as starting material. The haemin crystallized from ether in red-brown crystals of the typical haemin shape. The crystals showed distinct pleochroism; with polaroid as analyser they were brown when their long axis was parallel to the plane of polarization, and green when it was perpendicular to it. Recrystallization from acetic acid-sodium chloride yielded square plates.

Analyses.	Found.	Calc. for $C_{33}H_{35}O_4N_5FeCl$.
N	10·30	10·66

Reaction of Verdohaemochromogen with Methylamine.

If verdohaemochromogen is incubated with a 30 p.c. solution of methylamine, or refluxed with it, no changes comparable to those with ammonia occur. The solution remains green, and on treatment with acid, green bile pigment is obtained, while no acid resistant haemin can be discovered.

This excludes the possibility that our monoaza-haemins or those synthesized by Fischer (cf. below) are compounds with the group $>C=NH$ between pyrrole rings. The formation of such compounds might have been possible, if verdohaemochromogens contained a $>CO$ group between pyrrole rings as assumed by Libowitzky. Methylamine ought then to react as readily as ammonia with the formation of $>C=N.CH_3$ compounds. Methylamine cannot form aza-haemins, however, since all three hydrogen atoms of ammonia are required for the condensation.

The absorption bands of the monoaza-protohaematin compounds lie about 10 mμ closer to the red end of the spectrum than those of the corresponding meso compounds. The globin or ammonia monoaza-haemochromogens differ in the position of their absorption bands much more from the porphyrin-haemochromogens than do the pyridine monoaza-haemochromogens from the pyridine porphyrin-haemochromogens; e.g. ammonia ferromonoaza-mesoporphyrin 561 mμ, ammonia ferromesoporphyrin 546 mμ, $\Delta = 15$ mμ; pyridine ferromonoaza-mesoporphyrin 556 mμ, pyridine ferromesoporphyrin 550 mμ, $\Delta = 6$ mμ. This may be of importance in the search for aza-haematin compounds in biological material, where their presence may have been overlooked due to the application of pyridine for haemochromogen formation.

The similarity between monoaza-haematin and haematin compounds is evident. In both classes, for example, the pyridine ferrous compounds have the sharpest and highest absorption bands, and only the pyridine ferric compounds show indicator properties; and so on. The type of spectrum is also similar; a closer examination of the absorption of monoaza-haemochromogens reveals the existence of a weaker second band as in haemochromogens, with the only difference that the position of this band is closer to that of the main band, so that it cannot easily be ob-

TABLE 1.

Spectroscopic properties of monoaza-haematin compounds.

Compound.	Solution.	Colour.	Position of absorption bands in mμ. Proto.	Meso.	Remarks.
Ferroporphyrin (haem)	Ammonia 0·03 p.c.	Greenish slate, in artificial light purple	589	581	Weak band.
Pyridine ferroporphyrin (haemochromogen)	Dilute pyridine or alkali	Purple	564·2	556	Strong sharp band.
Ammonia ferroporphyrin	Ammonia 10 p.c.	Purple	571·9 (555)	561 (550)	Only in strong ammonia.
CO ammonia ferroporphyrin	Ammonia 10 p.c.	Bluish purple	570	—	Diffuse band.
Denatured globin ferroporphyrin	NaOH 1/5 N	Purple	583	561	Combined with denatured horse globin.
Ferrohaemoglobin (reduced hb.)	Phosphate buffer pH 6	Violet	591	—	Combined with native ox globin.
	dilute ammonia pH 9	Red	(591) weak 573	—	
CO ferrohaemoglobin	Phosphate buffer pH 6	Red	582	—	
Ferrihaemoglobin (methaemoglobin)	Phosphate buffer pH 6	Purple	568	—	The compound obtained by oxygenation of ferrohaemoglobin was spectroscopically not clearly distinguishable from ferrihaemoglobin.
	dilute ammonia pH 9	Purple	581 (542)	—	
Ferriporphyrin (alkaline haematin)	NaOH 1/5 N	Olive green	616 to 625	600 to 615	Variations in the position of these bands are due to their diffuse and asymmetrical nature.
Pyridine ferriporphyrin (parahaematin)	Aqueous pyridine or phosphate buffer pH 6	Greyish purple	566	—	Two different parahaematins exist; the red form, stable in neutral or weakly acid sol. is not much dissociated in 0·5 p.c. pyridine. One drop of 1 p.c. Na_2CO_3 transforms it into the green form, which is clearly different from uncombined ferriporphyrin. Similar observations in the protohaem series have been reported by Barron (1937).
	dilute Na_2CO_3 or 1/5 N NaOH	Pure green	610	600	
Ammonia ferriporphyrin (parahaematin)	Strong ammonia	Purple	566	551	Strong ammonia is required for combination. The green form is not so clearly distinguishable from uncombined ferriporphyrin as the pyridine compound.
	same and N/5 NaOH	Green	625	—	
Cyanide ferriporphyrin	0·02 p.c. NH_3, 5 mM KCN pH 10·4	Brown-red	575	561	
Cyanide ferroporphyrin	0·02 p.c. NH_3, 5 mM KCN $Na_2S_2O_4$	Greenish purple	575 (573)	563 —	The acid form was observed by Mr. H. F. Holden. Cf. Lemberg *et al.*, 1941, p. 336.
	same and phosphate buffer pH 6·4	Blue-green	603	—	

served as a separate band. The study of the absorption spectra of the monoaza-haemoglobin compounds cannot claim to be more than preliminary, but there appear to be noticeable divergencies from those of haemoglobin compounds.

Absorption spectra of haematin compounds derived from Fischer's monoimido-porphyrins have not been described. The haemochromogens of diaza-coproporphyrin ester (Fischer and Müller, 1937) show absorption bands similar to those reported above for the monoaza-haemochromogens.

Ultraviolet Absorption Spectra.

The absorption of monoaza-haem compounds in the ultraviolet was studied in collaboration with Mr. H. F. Holden. The instrument was a Hilger medium spectrograph with the Hilger echelon cell outfit, loaned to the Walter and Eliza Hall Institute, Melbourne, by the National Health and Medical Research Council of Australia.

TABLE 2.

	Monoaza-haem series.		Protohaem series.		
Type of compound.	Position of maximum in mμ.	ε mol.	Position of maximum in mμ.	ε mol.	Reference.
1. Ferriporphyrin	362	48,000	385	49,000	Hicks and Holden (1929).
2. Pyridine ferriporphyrin	396	78,000	—	—	
3. Pyridine ferroporphyrin	410	86,000	409	75,000	Holden and Lemberg. (1939).
4. Cyanide ferriporphyrin	410	45,000	422	88,000	Barron (1937).
5. Cyanide ferroporphyrin pH 8·8	426	58,000	436	88,000	Barron (1937).
6. pH 6·4	387	44,000*	—	—	
7. Ferri-haemoglobin pH 10	402·5	74,000	411	71,000	Hicks and Holden (1929).
8. pH 5·1	400	80,000	—	—	
9. Cyanide ferrihaemoglobin	413	62,000	—	—	
10. Ferrohaemoglobin	410	55,000	429·5	134,000	Hicks and Holden (1929).
11. CO ferrohaemoglobin	410	90,000	418	154,000	Hicks and Holden (1929).

* Unstable, associating. ε mol = M/c. d log I°/I, where I° = intensity of entering light, I = intensity of transmitted light, d = thickness of layer in cm., c = concentration in gm. per l, and M = molecular weight.

Stock A: 1·20 mg. monoaza-haemin in 0·3 ml. 0·5 N NH_3, H_2O to 10 ml.
Stock B: 1·32 mg. monoaza-haemin in 5·0 ml. aqueous pyridine, 20 p.c. by vol.

1. 1 ml. stock A to 5 ml. with water, pH 10·2. Monoaza-haemin about 0·04 mM.
2. 0·8 ml. stock B to 5 ml. with aqueous pyridine.
3. As 2 with 20 mg. $Na_2S_2O_4$.
4. 1 ml. stock A + 0·4 ml. 1/15 M KCN, water to 5 ml. pH 10·4. NH_3: 3 mM, cyanide 5·3 mM. Visible absorption band 575 mμ. The compound may be a cyanide ammonia ferriporphyrin, cf. Drabkin (1942).
5. 1 ml. stock A + 0·4 ml. 1/15 M KCN + 0·3 ml. 5 p.c. $Na_2S_2O_4$ in 0·5 N NH_3, water to 5·0 ml. pH 8·8 ammonia: 33 mM, cyanide: 5·3 mM. Visible absorption bands 576, 538 mμ.
6. 1 ml. stock A + 0·4 ml. 1/15 M KCN + 0·4 ml. 1/15 M KH_2PO_4 + 0·3 ml. 5 p.c. $Na_2S_2O_4$ in water, pH 6·4. ammonia: 3 mM, cyanide: 5·3 mM. Visible absorption band 603 mμ.
7. 1 ml. stock A + 1 ml. ox globin + 0·1 ml. 0·5 N NH_3, water to 5·0 ml. pH 10·0.
8. 1 ml. stock A + 1 ml. rabbit globin + 0·2 ml. 1 p.c. acetic acid, water to 5·0 ml. pH 5·07.
9. 1 ml. stock A + 0·4 ml. 1/15 M KCN + 0·4 ml. 1/15 M KH_2PO_4 + 1 ml. rabbit globin, water to 5·0 ml. pH 8·17.
10. 1 ml. stock A + 1 ml. ox globin + 0·5 ml. 0·5 N NH_3, water to 5·0 ml. pH 10·0.
11. 1 ml. stock A + 1 ml. ox globin + 0·5 ml. 0·5 N NH_3. CO passed in and 20 mg. $Na_2S_2O_4$ in 0·2 ml. 0·5 N NH_3 added, water to 5·0 ml. pH 9·5.

In Table 2 the ultraviolet absorption bands of various monoaza-haem compounds are compared with those of the corresponding protohaem compounds in position and strength. The data on the protohaem derivatives are taken from the literature. It is evident that the ultraviolet absorption spectra of both types of compounds are very similar, and that the typical Soret band is found in monoaza-haem compounds. A few monoaza-haem derivatives, most noticeably the reduced haemoglobin, have a somewhat lower maximal absorption than the corresponding protohaem compounds.

Monoaza-porphyrins.

Monoaza-haemin and monoaza-mesohaemin were found to be extraordinarily stable to acids. Concentrated sulphuric acid, which transforms haemin into haematoporphyrin, splits monoaza-haemin only to a very slight extent. The monoaza-haemins can, however, be transformed to monoaza-porphyrins by hydrazine hydrate in glacial acetic acid. The diaza-haemins have also been split in this way (Fischer and Müller, 1937).

Monoaza-mesohaemin treated in this way gave a small amount of mesoporphyrin, which was extracted from ether by 1 p.c. hydrochloric acid, and as main product the more weakly basic monoaza-mesoporphyrin, which was extracted from ether by 10 p.c. hydrochloric acid with blue-violet (in artificial light red-violet) colour. The hydrochloric acid solution showed a strong absorption band at 595 mμ and a weaker band at 551 mμ. By sodium acetate the aza-porphyrin was driven into ether with brown-purple colour and strong red fluorescence. In Table 3 the absorption bands of monoaza-mesoporphyrin in ether-acetic acid are compared with those of mesoporphyrin, and the bands of monoaza-aetioporphyrin (Fischer and Friedrich, 1936; Stern and Pruckner, 1937) with those of aetioporphyrin. It is seen that monoaza-mesoporphyrin differs from mesoporphyrin in exactly the same way as monoaza-aetioporphyrin from aetioporphyrin. The order of strength of the bands in the two aza-porphyrins is the same, the band in the red being the strongest (cf. Stern and Pruckner, 1937).

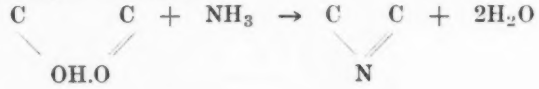
TABLE 3.

Absorption bands of porphyrins in ether-acetic acid in mμ.

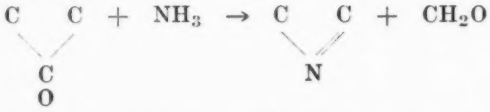
	Meso.	Aza-meso.	Δ.	Aetio.	Aza-aetio.	Δ.
I	623·5	614	− 9·5	619	610	− 9
Ia	597	588	− 9	596	588	− 8
II	569	562	− 7	566	560	− 6
III	529	534	+ 5	529	534	+ 5
IV	497·5	502	+ 4·5	497	501	+ 4

DISCUSSION.

Under the mild conditions in which monoaza-haemochromogen is formed from verdohaemochromogen the reaction:



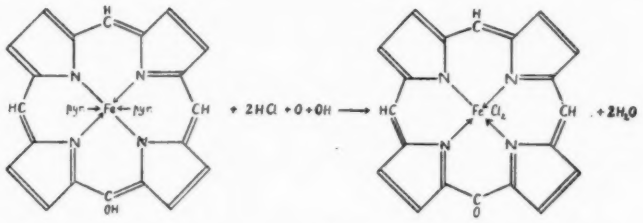
is more likely than the reaction



which would have to be assumed if verdohaemochromogens had the structure assumed for them by Libowitzky.

Libowitzky bases his proposed structure mainly on analytical data of coproester verdohaemin, a green haemin-like compound with two chlorine atoms, obtained from verdohaemochromogen. The analyses suggest the presence of nine,

not of ten oxygen atoms, which would be required for the open ring formula. The second chlorine atom is assumed to be present in the form of a hydrochloride. This is unlikely, since no haemin has been observed to form a hydrochloride. The formation of a compound with a keto group between two pyrrolic nuclei from a ferrohydroxyporphyrin would have to be accompanied by a profound alteration of the type of iron linkage, which may be envisaged in the following way:

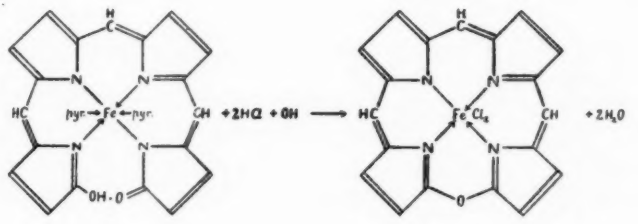


The iron in the ferric dichloro compound would have to be bound to the porphyrin ring by one, not by two covalent linkages and by three instead of two co-ordinate linkages.

Libowitzky's analyses, however, fit even better a similar formula with one carbon atom less and a ring closed by an oxygen atom (cf. Formula II below). This formula is in better agreement with the easy transformation into biliverdins, for instance, by the successive action of alkali and acid.

The formation of verdohaemochromogen on the basis of a CO closed ring is not attempted by Libowitzky. Analytical evidence (unpublished results) show that verdohaemochromogen contains two pyridine molecules. A magnetochemical study by Dr. Mellor at the Department of Chemistry, Sydney University, has shown that it has certainly not more than one free electron on the iron atom; it may contain none, since verdohaemochromogen is unstable and rapidly transformed into strongly paramagnetic substances.

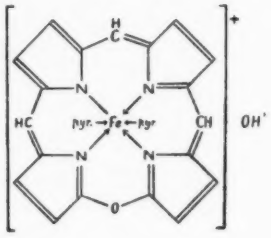
The most likely explanation of all the facts appears to be that verdohaemochromogen has an open ring system, but when oxidized in the presence of hydrochloric acid, undergoes an intramolecular rearrangement with condensation to an oxygen-closed ring system:



I. II.

This can explain why mild treatment of verdohaemochromogen leads more readily to the formation of biliverdins than treatment with hot hydrochloric acid.

An alternative explanation is that verdohaemochromogen itself possesses an oxygen-closed ring system. It has then to be formulated as III, i.e. as a ferrous compound, but not as a typical haemochromogen. In favour of such a formula may be adduced the reversible reduction of verdohaemochromogen by hyposulphite in alkaline solution to a yellow compound, which in its absorption spectrum resembles bilirubin, but which is converted by acids into a yellow compound distinct from bilirubin.



III.

The transformation of haem to verdohaem to aza-haem is accompanied by remarkable changes in the stability of the iron linkage. Verdohaematin is very unstable, aza-haematin more stable than haematin itself. The explanation of the great stability of aza-haemins is to be sought in the steric conditions of the ring. The distance between the two carbon atoms 1 and 3 in $\overset{1}{C} - CH = \overset{3}{C}$ can be estimated at 2·53 Å, while linkage by N in $\overset{1}{C} - N = \overset{3}{C}$ decreases this distance to 2·30 Å. Robertson (1937), (cf. also Barret *et al.* 1936), has shown that even in the phthalocyanines with four $C - N = C$ linkages the space in the middle of the molecule is still 0·05 Å. wider than the radius of the iron atom. The contraction of the ring by N replacing CH will therefore tend to increase the stability of the iron linkage.

If the open ring formula of verdohaemochromogens is correct, the explanation of their instability is also stereochemical. The formation of the $\overset{1}{C}.OH.O = \overset{3}{C}$ linkage with a hydrogen linkage between oxygen atoms involves an increase of the distance of carbon atoms 1 and 3 from 2·53 Å. in haemin to 4·74 Å. in verdohaematin. With or without deviation of the molecule from planar arrangement this extension of the ring would cause a great strain on the iron bonds.

If the formula with an oxygen closed ring is correct, the instability of verdohaematin iron must be explained by the peculiar type of the iron linkage with one covalent bond, rather than by stereochemical factors.

SUMMARY.

By treatment with ammonia verdohaemochromogens are transformed into monoaza-haemochromogens. The reaction proceeds under mild conditions at room temperature and in the absence of oxygen.

Monoaza-haemin and monoaza-mesohaemin have been prepared, and the spectroscopic properties of various compounds with nitrogenous bases, globin and cyanide have been studied. The ultraviolet absorption spectra of monoaza-haem

compounds show typical Soret bands, similar in strength and position to those of the corresponding porphyrin compounds.

Iron can be removed from monoaza-haemins by hydrazine hydrate and glacial acetic acid, not by conc. sulphuric acid. The monoaza-porphyrins are identified with the "monoimido-porphyrins" synthesized by Fischer. The reaction opens the first well-defined way to these substances and confirms their structure.

The significance of these observations for the problem of the structure of verdohaemochromogens is discussed, and an explanation is proposed for the great stability of the iron linkage in aza-haemins and its instability in verdohaematins.

REFERENCES.

Barron, E. S. G. (1937): J. biol. Chem., 121, p. 285.
Drabkin, D. L. (1942): *Ibid.*, 142, p. 855.
Endermann, F. and Fischer, H. (1939): Lieb. Ann., 538, p. 172.
Fischer, H., Haberland, W. and Müller, A. (1935): *Ibid.*, 521, p. 122.
Fischer, H. and Friedrich, W. (1936): *Ibid.*, 523, p. 154.
Fischer, H. and Müller, A. (1937): *Ibid.*, 528, p. 1.
Fischer, H. and Endermann, F. (1937): *Ibid.*, 531, p. 245.
Fischer, H. and Libowitzky, H. (1938): Z. physiol. Chem., 251, p. 198.
Helberger, J. H. (1937): Lieb. Ann., 529, p. 205.
Hicks, C. S. and Holden, H. F. (1929): Austral. J. exp. Biol., 6, p. 175.
Holden, H. F. and Lemberg, R. (1939): *Ibid.*, 17, p. 133.
Lemberg, R. (1935): Biochem. J., 29, p. 1322.
Lemberg, R., Cortis-Jones, B. and Norrie, M. (1938a): *Ibid.*, 32, p. 149.
Lemberg, R., Cortis-Jones, B. and Norrie, M. (1938b): *Ibid.*, 32, p. 171.
Lemberg, R., Legge, J. W. and Lockwood, W. A. (1941): *Ibid.*, 35, p. 328.
Libowitzky, H. and Fischer, H. (1938): Z. physiol. Chem., 255, p. 209.
Libowitzky, H. (1940): *Ibid.*, 265, p. 135.
Metzger, W. and Fischer, H. (1936): Lieb. Ann., 527, p. 1.
Stern, A. and Pruckner, F. (1937): Phys. Chem. (A), 178, p. 420.
Stier, E. (1942): Z. physiol. Chem., 273, p. 47.
Warburg, O. and Negelein, E. (1930): Ber. Dtsch. chem. Ges. (B), 63, p. 1816.

ANTIBACTERIAL SUBSTANCES PRODUCED BY MOULDS

4. THE DETECTION AND OCCURRENCE OF SUPPRESSORS[1] OF PENICIDIN ACTIVITY

by NANCY ATKINSON AND NEVILLE F. STANLEY[2]

(From the Institute of Medical and Veterinary Science, Adelaide).

(Accepted for publication 9th September, 1943.)

During our earlier work with penicidin we observed that in certain culture media the activity of our preparations was lowered. We therefore investigated the ability of various media and other substances to "suppress" the action of penicidin. "Suppressors" of other antibacterial substances have been described. According to McLeod and Mirick (1942) "sulphonamide-inhibitors" occurred in culture media and interfered with the estimation of bacteriostatic activity. Working with a strain of pneumococcus Type I and sulphapyridine they found the following variations in bacteriostatic titre:

Medium.	Highest dilution of sulphapyridine inhibiting growth.
Peptone broth + 2 p.c. rabbit serum	1/5,000
Charcoal-adsorbed peptone broth + 2 p.c. rabbit serum	1/20,000, partial 1/50,000
Liver infusion + 2 p.c. rabbit serum	1/500,000, partial 1/1,000,000

They did not isolate the substance responsible for the reduction in titre.

Green and Bielschowsky (1942) found "sulphonamide-inhibitors" in bacterial extracts and traced the activity to the presence of p-aminobenzoic acid which exerted a specific anti-sulphonamide action. Green (1940) denied that media possessed specific anti-sulphonamide activity, and concluded that "the nature of the medium affected the issue only by conditioning the rate of growth." Bearing in mind these observations we carefully controlled our experiments to avoid mistaking a non-specific growth-promoting effect for specific anti-penicidin activity. As the media which we tested all gave different penicidin titres but supported the growth of *Bact. typhosum* equally well we do not think that Green's statement applies to our results.

Fildes (1940) suggested that the growth-inhibiting effect of anti-bacterial substances might be ascribed to a specific poisoning of some essential metabolic process by interference with the catalyst (enzyme) or the "essential metabolite" (substrate). This interference might take place in more than one way. The antibacterial substance might combine with the essential metabolite to form a product useless to the bacterium, or it might compete with the metabolite for the enzyme involved. In either case excess metabolite should be capable of specifically suppressing the antibacterial action and might show some quantitative relationship to the antiseptic.

According to Fildes' theory, (1) the specific suppressor of an antibacterial agent should be an essential metabolite; (2) the finding of a specific suppressor should provide information on the mode of action of the suppressed antiseptic;

[1] We have introduced the term "suppressor" for materials which reduce the activity of antibacterial substances. Other workers have used "inhibitor" which is also applied to the antibacterial substances themselves. By using "suppressor" we avoid the awkward situation arising out of inhibitors inhibiting inhibitors.

[2] Working with a grant from the University of Adelaide.

(3) previously unrecognized essential metabolites might be found which would indicate essential reactions previously unknown in the cell.

The theory might also be applied to the discovery of new anti-bacterial substances. In order to interfere competitively in an enzyme system a substance must possess a chemical structure resembling that of the substrate; therefore substances structurally related to an essential metabolite might show antibacterial activity. McIlwain (1942) is investigating this field and has shown that certain "metabolite analogues" possess antibacterial power. For instance, pantoyl taurine, analogue of pantothenic acid, inhibited *C. diphtheriae,* and pantothenic acid specifically reversed the inhibition.

A study of penicidin suppressors might provide information on the mode of action of penicidin and a contribution to the theory of antiseptic action.

EXPERIMENTAL WORK.

Penicidin-suppressors were detected readily by their ability to reduce the antibacterial titre of the substance. To determine this titre serial two-fold dilutions of the penicidin solution were made in a suitable liquid medium; after inoculation of the test organism and incubation for 24 hours at 37° C., the tubes were examined for turbidity. In adopting this technique for the detection of suppressors we standardized the two more important variables: the inoculum and the culture medium. Concerning the inoculum, we considered the following points: (1) the number of organisms introduced per ml. of medium should be as nearly constant as possible; (2) the inoculum should always be prepared from a culture of similar age on the same medium; (3) care should be taken to avoid introducing with the inoculum any extraneous material such as portions or extracts of culture media possibly containing inhibitors or growth-promoting substances; (4) the diluting fluid should neither encourage growth nor cause the death of the organisms; (5) the sensitivity of the test-organism to antibacterial action should be confirmed regularly. Regarding the culture medium, we sought one which was free from suppressors and capable of supporting satisfactorily the growth of the test-organism.

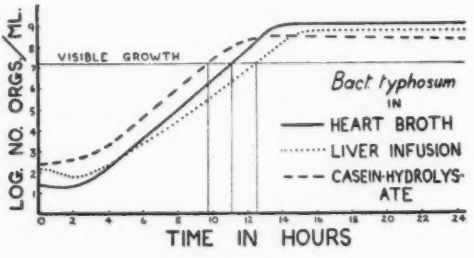


Fig. 1.

The inoculum. *Bact. typhosum* was the test-organism; the diluting fluid was Ringer's solution in which, during several hours at room temperature, suspensions of *Bact. typhosum* showed little alteration as judged by viable counts. The organisms were rapidly washed off an agar slope which had been incubated for 18–24 hours at 37° C. after inoculation with a loopful of an 18 hours' broth culture. By comparison with an opacity standard this suspension was adjusted to approximately 10^9 organisms per ml. and from it serial ten-fold dilutions were made. The standard inoculum was 0·1 ml. of the dilution containing approximately 10^3 organisms per ml. (approximately 100 organisms), added from a dropping pipette (1 drop = 0·02 ml.) to every 5 ml. of medium. On account of the high dilution (1/1,000,000) of the original suspension we assumed that this inoculum did not contain any appreciable amount of material from the original culture medium. All inoculations were made within two hours of preparation of the suspension which was kept at room temperature.

The sensitivity of the strain of *Bact. typhosum* was checked every fortnight by titration against phenol in nutrient broth.

The medium. We wished to find a medium which satisfactorily supported growth of *Bact. typhosum* and did not contain penicidin-suppressors. The media selected for investigation were

nutrient broth, nutrient broth adsorbed with charcoal (which partly removed "sulphonamide-inhibitors" according to McLeod and Mirick, 1942), liver infusion (which, according to McLeod, 1940, did not contain "sulphonamide-inhibitors"), and the synthetic medium described by McLeod (1940), with the addition of 3 p.c. casein-hydrolysate (McIlwain, 1939).

The media were first tested for ability to support growth of *Bact. typhosum*. In a preliminary trial 5 ml. of each medium were inoculated and the resulting turbidities compared after 18 hours at 37° C. An approximately equal turbidity appeared in nutrient broth and liver infusion but slightly less in the charcoal-adsorbed broth; the turbidity in the casein-hydrolysate medium was only about half that produced in broth.

More detailed information was obtained from viable counts made at intervals; growth curves were constructed and are shown in Fig. 1. The charcoal-adsorbed broth was not included.

Judging by the growth curves, all three media supported satisfactory growth of the standard inoculum. The casein-hydrolysate medium showed a very short lag phase followed by rapid growth in the logarithmic phase; visible turbidity appeared earlier than in the other media. The maximum population was smaller than that achieved in the other media; the final turbidity was therefore less, as we observed in our preliminary test; in spite of this result we concluded from the general growth curve that this medium was satisfactory for titrations.

The media were tested next for penicidin-suppressors. A titration was performed in each medium and the titres compared; differences in titre indicated variation in content of suppressor. Early work had indicated that penicidin was gradually inactivated during incubation in broth. To detect this type of suppression one set of dilutions of penicidin in each medium was incubated for 24 hours at 37° C. before inoculation; the tubes were then inoculated and incubated. A decrease in titre compared with the previous titration (without preliminary incubation) indicated the presence of these "slow suppressors". The results of these titrations are given in Table 1.

TABLE 1.

Penicidin activity in various media.

Medium.	Preliminary incubation.	Penicidin dilutions. 1/40	1/80	1/160	1/320	1/640	1/1280	1/2,560	1/5,120	1/10,240
Nutrient broth	None	—	—	—	—	—	—	+	+	+
	24 hours	—	—	—	+	+	+	+	+	+
Charcoal adsorbed broth	None	—	—	—	—	—	—	±	+	+
	24 hours	—	—	—	+	+	+	+	+	+
Casein-hydrolysate medium	None	—	—	—	—	—	—	—	—	+
	24 hours	—	—	—	—	—	—	±	+	+
Liver infusion	None	—	—	—	—	—	—	—	+	+
	24 hours	—	+	+	+	+	+	+	+	+

+ = turbidity equal to that in medium with no penicidin. — = no turbidity.

Every medium showed a fall in titre after preliminary incubation; therefore all contained slow suppressors. The greatest decrease occurred in liver infusion: from 1/2,560 to 1/40. The casein-hydrolysate medium contained the least suppressor as it gave the highest titre and the smallest decrease with preliminary incubation. On this account we selected this medium for subsequent work.

The test for penicidin-suppressors. The material to be tested was mixed with an equal quantity of penicidin solution, and the mixture incubated at 37° C. for 24 hours to allow slow suppressors to act; it was then titrated in casein-hydrolysate medium. A similar mixture, which was freshly prepared, was titrated at the same time. A control, consisting of penicidin solution mixed with an equal quantity of water, was also titrated with and without preliminary incubation. A disadvantage of the method lay in the introduction into the medium of falling concentrations of the test-material along with the penicidin; a growth-promoting or inhibiting effect might result; such an effect, however, could be readily detected by comparison of the test with the control, and provided no serious obstacle to the interpretation of the results. An advantage of the method lay in the roughly quantitative nature of the results, which indicated the relative amounts of suppressor in the test-substance.

The occurrence of penicidin-suppressors. Various materials were tested and the results are given in Table 2.

The control in water showed that the activity of penicidin was not affected by incubation at 37° C. for 24 hours.

In the tests without preliminary incubation the casein-hydrolysate, yeast extract, peptone, liver infusion and the various sera did not cause any alteration in the antibacterial titre. After

preliminary incubation all these substances with the exception of yeast extract, reduced the titre: the three sera were most active causing a marked reduction from 1/1,024 to less than 1/4. The casein-hydrolysate was also active, reducing the titre from 1/1,024 to 1/8.

Sodium thioglycollate acted differently from the sera, extracts, etc.; it altered the titre without preliminary incubation, which caused no further alteration. There was a marked decrease in the titre: from 1/1,024 to 1/64.

TABLE 2.

Tests for Penicidin Suppressors.

Material tested.	Preliminary incubation.	Penicidin dilutions. 1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024	1/2,048	1/4,096	1/8,192
Water (control)	None	—	—	—	—	—	—	—	—	—	+	+	+
	24 hours	—	—	—	—	—	—	—	—	—	+	+	+
Casein hydrolysate	None	—	—	—	—	—	—	—	—	—	+	+	+
	24 hours	—	—	+	+	+	+	+	+	+	+	+	+
Yeast extract	None	—	—	—	—	—	—	—	—	—	+	+	+
	24 hours	—	—	—	—	—	—	—	—	—	+	+	+
Sodium thioglycollate (10 p.c.)	None	—	—	—	—	+	+	+	+	+	+	+	+
	24 hours	—	—	—	—	+	+	+	+	+	+	+	+
Peptone 1 p.c.	None	—	—	—	—	—	—	—	—	—	+	+	+
	24 hours	—	—	—	—	—	—	—	—	+	+	+	+
Horse serum	None	—	—	—	—	—	—	—	—	—	+	+	+
	24 hours	+	+	+	+	+	+	+	+	+	+	+	+
Sheep serum	None	—	—	—	—	—	—	—	—	—	+	+	+
	24 hours	+	+	+	+	+	+	+	+	+	+	+	+
Human serum	None	—	—	—	—	—	—	—	—	—	+	+	+
	24 hours	+	+	+	+	+	+	+	+	+	+	+	+
Liver infusion	None	—	—	—	—	—	—	—	—	—	+	+	+
	24 hours	—	—	—	—	—	—	—	—	+	+	+	+

We are dealing therefore with two distinct types of suppression, namely, the slow inactivation which appeared only after a period of preliminary reaction with the penicidin (effect of sera, extracts, etc.), and the suppression produced by sodium thioglycollate which was immediately effective and unaltered by preliminary incubation.

DISCUSSION.

Many complex substances of animal or vegetable origin contain slow penicidin-suppressors which after a suitable preliminary period of reaction with the penicidin can be detected by their depressing effect on the antibacterial titre. The most active substance tested was serum which proved equally effective whether obtained from man, horse, or sheep. The effect was not due to non-specific growth-stimulation because control tests, without a period of preliminary reaction, gave the same titre as the penicidin alone.

Slow penicidin-suppressors were also found in various culture-media, of which the synthetic medium of McLeod (1940) with addition of 3 p.c. casein-hydrolysate contained the least and supported satisfactory growth of the test-organism, *Bact. typhosum*. We used this medium in detecting penicidin-suppressors. However it was not ideal, as slow penicidin-suppressors were present in the undiluted casein-hydrolysate of which a concentration of 3 p.c. was present in the medium.

Sodium thioglycollate acted differently from the slow suppressors; it inactivated penicidin without any preliminary incubation.

We can explain the action of the slow suppressors as a chemical reaction, or an adsorption effect proceeding at a slow rate between the penicidin and some substance

in the serum, etc.; as a result the penicidin molecule becomes altered in such a way that the group responsible for antibacterial activity is destroyed. The substance neutralizing the penicidin-activity may not be the same in all materials containing suppressor. At present we are fractionating some of these materials in an effort to locate the suppressors; so far we have obtained an active fraction from peptone by alcohol-precipitation, and have removed the activity from horse serum by dialysis followed by filtration. We also have some evidence for the slow rate of inactivation; we found that dilutions of penicidin in liver infusion ready for titration, required a preliminary incubation for six hours at 37° C. to produce a two-fold decrease in titre.

The action of sodium thioglycollate may be explained in several ways. A rapid, specific chemical reaction with penicidin may occur and produce an inert compound; this action would be comparable with that of thiolacetic acid and other SH compounds on the antibacterial activity of mercury (Fildes, 1940), which is rendered inactive by reaction with the SH group to form a substance no longer capable of combining with the SH of essential metabolites or cells. Or an essential enzyme reaction may be specifically affected and the action resemble that of p-aminobenzoic acid in suppressing the sulphonamides, not by chemical combination, but by competitive interference in an essential enzyme reaction (Woods, 1940). Or again, thioglycollate may act non-specifically by affecting the Eh of the medium.

Further work is proceeding to determine which theory is most tenable. We have some evidence that increase in reducing potential alone cannot suppress penicidin.

Although penicidin does not contain sulphur (SH) it resembles thioglycollate in possessing reducing power. In this resemblance, at least, we have a common characteristic which may be partly responsible for results observed. We hope that further work on the suppression of penicidin activity will throw some light on the mechanism of its anti-bacterial action.

SUMMARY.

A test for "penicidin-suppressors" has been described.

The suppressors have been demonstrated in culture-media, serum and various substances of animal and vegetable origin.

These substances act slowly, but in 24 hours at 37° C. produce a marked suppression.

The activity of penicidin is also suppressed by sodium thioglycollate. The possible mechanism of this reaction is discussed.

REFERENCES.

Fildes, P. (1940): Brit. J. ex. Path., 21, p. 67.
Green, H. N. (1940): *Ibid.*, 21, p. 38.
Green, H. N. and Bielschowsky, F. (1942): *Ibid.*, 23, p. 1.
McIlwain, H. (1939): *Ibid.*, 20, p. 330.
McIlwain, H. (1942): Biochem. J., 36, p. 417.
McLeod, C. M. (1940): J. exp. Med., 72, p. 217.
McLeod, C. M. and Mirick, G. S. (1942): J. Bact., 44, p. 277.
Woods, D. D. (1940): Brit. J. exp. Path., 21, p. 74.

ANTIBACTERIAL SUBSTANCES PRODUCED BY MOULDS

5. THE MECHANISM OF THE ACTION OF SOME PENICIDIN SUPPRESSORS[1]

by NANCY ATKINSON AND NEVILLE F. STANLEY[2]

(From the Institute of Medical and Veterinary Science, Adelaide).

(Accepted for publication 9th September, 1943.)

In a previous paper (Atkinson and Stanley, 1943) we recorded the occurrence of penicidin-suppressors in a variety of materials. Some substances required preliminary incubation with penicidin and were called "slow suppressors;" they are not dealt with here. This paper concerns the mode of action of the other type of suppressor which was unaffected by preliminary incubation. Thioglycollic acid was the only substance of this type reported in the previous work, in which we speculated upon the possible mechanism of its action. We suggested as alternatives specific chemical reaction, competitive enzyme inhibition, or non-specific effect due to strong reducing conditions. The thioglycollate molecule is unremarkable except in its possession of an SH group which may be responsible for its anti-penicidin activity. We therefore tested various SH compounds as well as other reducing agents. The results of these experiments pointed to a specific action of the SH group.

EXPERIMENTAL WORK.

The test of penicidin-suppression. We used the method previously described, with the modification that two-fold dilutions of penicidin were made up in the casein-hydrolysate medium. To every tube we added a given concentration of the test-substance. Control tests without penicidin demonstrated the ability of *Bact. typhosum* to grow satisfactorily in the medium containing the given concentrations of the test-substance.

Tests with SH compounds. We tested thioglycollic acid, glutathione and cysteine HCl. The thioglycollic acid was prepared in M/10 concentration in water, neutralized, made up to M/20 and used immediately. The glutathione and cysteine HCl were dissolved in water to give a solution M/100 and were used at once. The results of these tests are given in Table 1.

TABLE 1.

Experiments with SH compounds.

	Penicidin dilutions.										No
Test compound.	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024	1/2,048	penicidin.
Thioglycollate											
M/200	−	+	+	+	+	+	+	+	+	+	+
M/500	−	−	−	−	+	+	+	+	+	+	+
M/1,000	−	−	−	−	−	−	−	+	+	+	+
Glutathione											
M/1,000	−	−	−	+	+	+	+	+	+	+	+
M/10,000	−	−	−	−	−	−	−	+	+	+	+
M/50,000	−	−	−	−	−	−	−	−	±	+	+
Cysteine											
M/1,000	−	−	−	−	−	+	+	+	+	+	+
M/10,000	−	−	−	−	−	−	−	−	+	+	+
None	−	−	−	−	−	−	−	−	−	+	+

+ = turbidity equal to that in medium without penicidin.

[1] For the meaning of this term see No. 4 of this series of articles.

[2] Working with a grant from the University of Adelaide.

The three SH compounds suppressed penicidin to an extent dependent upon their concentration. A comparison of the results for all compounds at the same concentration (M/1,000) shows that glutathione is the most active, as it reduced the penicidin titre from 1/1,024 to 1/16; cysteine reduced the titre to 1/64, and thioglycollate produced a still smaller reduction to 1/256.

The effect of thioglycollate differed markedly from that of glutathione or cysteine. The suppressing activity of thioglycollate fell off rapidly with dilution; at M/200 the penicidin-titre was reduced to 1/8, but at M/1,000 the titre was 1/256. Thus with five-fold dilution of M/200 thioglycollate, its anti-penicidin activity was reduced 32 times. With glutathione and cysteine the suppression of penicidin fell off fairly evenly with dilution; glutathione M/1,000 reduced the penicidin titre to 1/16; at M/10,000 it reduced the titre to 1/256, which is close to the calculated titre of 1/160, a dilution which did not occur in our test. Cysteine behaved in the same way.

Fildes (1940) working on the anti-mercurial action of SH compounds, noted that glutathione was more active than thiolacetic acid. Each of these compounds possessed one SH group per molecule and should be equally potent if the activity was entirely due to SH. Fildes, accepting the specificity of action of the SH group, attributed the difference in potency of thiolacetate and glutathione to a difference in their ability to dissolve and react with a mercury-ammonium-compound which formed in his test-medium on addition of $HgCl_2$. We do not think this explanation is applicable to the difference between thioglycollate (which may be classed with thiolacetate) and glutathione in supressing penicidin.

Tests with compounds not containing SH. We tested the following compounds, all of which possessed reducing power: ascorbic acid M/2,000 and M/10,000, pyrogallol M/6,000, hydrazine hydrate (neutralized) M/1,000, $FeSO_4$ M/500 and M/200. At the concentrations tested, none of these compounds acted as penicidin-suppressors. We therefore concluded that the SH group acted specifically in suppressing penicidin.

Effect of anaerobic incubation. Two titrations of penicidin were set up; one was incubated anaerobically in the McIntosh and Filde's jar and the other was incubated in the usual way. Both tests gave the same titre and showed that non-specific reduction did not suppress penicidin.

Tests for chemical reaction between SH compounds and penicidin. The SH group can be detected by the nitroprusside test. If penicidin reacted with the SH group, the nitroprusside test should be negative in a mixture containing excess penicidin. We mixed thioglycollate M/500 or glutathione M/1,000 with dilutions of penicidin corresponding to those used in the test for penicidin-suppression. From the results of the supression test we knew which mixtures contained excess penicidin (i.e. those which had shown no growth). We tested each mixture with nitroprusside and all gave a positive reaction. On allowing the tests to stand for a few minutes, however, the pink colour rapidly faded in the tubes containing the strongest penicidin, while the other tubes remained positive. We concluded that a lower concentration of SH was present in the tubes which faded and that therefore some of the SH compound had reacted with the penicidin, the concentration of which conditioned the degree of reaction.

These tests were made immediately after mixing. In case the reaction proceeded slowly another set of mixtures was incubated at 37° C and tested after 30 minutes, one hour and two hours. The nitroprusside test was positive each time; and it faded quickly as before in the strong solutions of penicidin.

DISCUSSION.

SH compounds suppressed penicidin while other reducing substances or anaerobic conditions were without effect. The SH group therefore acted as a specific penicidin-suppressor. Our tests showed a roughly quantitative relation between concentration of glutathione or cysteine and the amount of penicidin suppressed. This relationship suggests that the mechanism of suppression is either a chemical reaction or a competitive enzyme inhibition. The nitroprusside test showed that a certain amount of SH disappeared in the presence of excess penicidin. On this account we favour the theory of inactivation of penicidin by chemical reaction with the SH group. The products of the reaction could be either addition compounds of penicidin and SH substance or reduced penicidin and oxidized S substance. The latter seems the more likely, and if we accept it we must postulate that reduced penicidin no longer possesses antibacterial activity. As yet we have no evidence on this point. It is hard to see why other reducing agents do not suppress penicidin if reduction is the only factor involved; but it is possible that the chemical nature of penicidin makes it susceptible to reduction only under certain conditions which are supplied by the SH group.

We cannot yet exclude the possibility of competitive enzyme inhibition.

The difference in activity of the various SH compounds leads us to believe that the structure of the molecule is also important. It is surely significant that the most active compound is glutathione, a substance with particular physiological activity.

The suppression of penicidin by SH compounds suggests a theory of the mechanism of the antibacterial action of penicidin. Fildes (1940) has shown that mercurial antiseptics are specifically suppressed by SH compounds having little or no effect upon many other types of anti-bacterial substance. By analogy with his explanation of the mechanism of the antibacterial action of mercury, it is possible that the action of penicidin resembles that of mercury and interferes with the SH of essential metabolites or cells, thus rendering this group useless for cell metabolism.

SUMMARY.

Penicidin is suppressed specifically by SH compounds. It is suggested that the mechanism of this suppression is a chemical reaction between the penicidin and the SH group.

REFERENCES.

Atkinson, N. and Stanley, N. F. (1943): Austral. J. exp. Biol., 21, p. 249.
Fildes, P. (1940): Brit. J. exp. Path., 21, p. 67.

OBSERVATIONS ON *ANOPHELES ANNULIPES* WALK. AS A POSSIBLE VECTOR OF MALARIA

1. THE RELATIVE SUSCEPTIBILITY OF *AN. ANNULIPES* AND *AN. PUNCTULATUS* VAR. *MOLUCCENSIS* SW. TO EXPERIMENTAL INFECTION WITH MALARIA PARASITES

by F. H. S. ROBERTS

(A.A.M.C., Australia).

(Accepted for publication 2nd September, 1943.)

INTRODUCTION.

Heydon in 1942 (unpublished) showed *An. punctulatus* var. *moluccensis* to be the important vector of benign tertian malaria in the Cairns area. Previous investigations by the same worker (Heydon, 1923) had implicated this species as a vector of both B.T. and M.T. malaria at Rabaul, while the Dutch workers demonstrated that it was the chief vector in the vicinity of Merauke, Dutch New Guinea. Odd cases of indigenous malaria, however, have been reported from time to time in areas in Australia where *An. moluccensis* does not exist, and suspicion has on these occasions been directed against *An. annulipes*. Moreover, Heydon, some years ago in Sydney, was able to infect this species experimentally with malaria parasites, and in a Western Australian hospital it has been employed for the transmission of *P. vivax* to G.P.I. patients (Bentley, Thompson and Williams, 1930). On the other hand, it has never been associated with an epidemic of malaria in Queensland, from Townsville south, or in New South Wales, although it has certainly had opportunities to become infected. Observational evidence, in fact, strongly suggests that it is at best a poor natural vector. One cannot be assured that it will always remain so, especially if presented with large numbers of gametocyte carriers in areas where it is abundant, and it was felt that more precise knowledge was needed about its potentialities, in order to assess whether the risk is a real one or not.

Several factors combine to determine whether or not a given Anopheline may be a possibly dangerous vector of malaria, namely:

1. Is the species hospitable to infection, i.e. is it capable of rearing the parasites to the sporozoite stage? How does it compare with a known vector in this respect?
2. What is its avidity for human blood?
3. Does it habitually come in close contact with man; and, especially, does it frequent his habitations?
4. Does it occur in large numbers in the area under consideration?
5. Are there any conditions associated with its biology (e.g. its length of life) or habits which may prevent sporozoite development in nature?

The second and third of these questions are of by far the greatest practical importance; but they require a considerable time to study in any adequate detail, and only the first is considered in this paper, although a few incidental observations on feeding habits and survival in captivity are also recorded.

EXPERIMENTAL.

Infectivity of gametocyte carriers and the extrinsic factors affecting development vary so enormously, that it is a basic essential of all work of this kind, that the experimental batches

under comparison have precisely the same opportunities to acquire an infection, and are kept under precisely identical conditions throughout the experimental period. The following methods were adopted:

i. Larvae of *An. annulipes* and *An. moluccensis* were collected in the field and reared to the adult stage in the laboratory in Cairns. At first they were placed in tap water and fed on yeast. The mortality among those reared in this manner was extremely low, but the subsequent adults were short-lived and did not bite at all well. This was remedied by rearing the larvae in frequent changes of their natural water, plus the assistance of a little yeast as food.

ii. Equal numbers of both species of the same age were placed together in the one cage for feeding. The age of the lots used varied from 1–2 to 3–4 days. These were then transported to a hospital on the Atherton Tableland and each batch was fed once on a selected gametocyte carrier for a period of approximately 4 hours.

iii. After feeding, the engorged females were removed and identified, all engorged females of both species, which had fed together, being thereafter maintained under exactly identical conditions until dissected. The period between engorgement and dissection ranged from 10 to 14 days, all fed females from each batch being examined on the same day.

Experiments with Plasmodium vivax.

Five tests involving 187 adults of each species have been carried out to date. The results are summarized in Table 1.

TABLE 1.

Period from feeding to dissection, 10 days. Age when fed, 1 to 2 days.

Species.	Total adults used.	Total adults fed.	P.c. adults fed.	No. fed adults survived to dissection.	P.c. fed adults survived to dissection.	No. adults infected. Glands.	Gut only.	P.c. infected glands.
An. annulipes	187	79	42	43	54	15	2	35
Ann. moluccensis	187	69	37	41	60	12*	3	31

* 2 sets of glands lost on dissection.

An analysis of the intensity of infection found in the two species is shown in Table 2.

TABLE 2.

Species.	Gland infection. Heavy. No.	Heavy. P.c.	Light. No.	Gut infection.* Heavy. No.	Heavy. P.c.	Light. No.
An. annulipes	5	33	10	8	47	9
An. moluccensis	2	17	10	6	40	9

* More than 5 sporulating oocysts = heavy; less than 5 sporulating oocysts = light.

Experiments with Plasmodium falciparum.

In all, nine tests have been carried out with malignant tertian, involving 348 adults of each species. The results are summarized in Table 3 below.

TABLE 3.

Period from feeding to dissection, 10 to 14 days. Age when fed, 1 to 3 days.

Species.	Total adults used.	Total adults fed.	P.c. adults fed.	No. fed adults survived to dissection.	P.c. fed adults survived to dissection.	No. adults infected. Glands.	Gut only.	P.c. infected. glands.
An. annulipes	348	182	52	64	35	33*	5	53
An. moluccensis	348	117	34	51	44	22*	8	45

* 2 sets of glands of *An. annulipes* lost on dissection. 1 set of glands and 1 adult of *An. moluccensis* lost on dissection.

An analysis of the degree of gland and gut infection is shown in Table 4.

TABLE 4.

Species.	Gland infection. Heavy. No.	Heavy. P.c.	Light. No.	Gut infection. Heavy. No.	Heavy. P.c.	Light. No.
An. annulipes	15	45	18	24	66	14
An. moluccensis	9	41	13	15	50	15

Survival and Feeding Habits in Captivity.

Summing the results set out in Tables 1 and 3, it will be seen that 107, or 41 p.c., of 261 *An. annulipes* which fed survived the 10–14 days to dissection, while 92, or 49 p.c., of 186 *An. moluccensis* survived the same period. These figures do not suggest any great difference in survival rate, but it is to be remembered that they give no indication of probable longevity in nature, nor do they suggest how long either species might survive after sporozoites had developed.

As regards avidity for human blood, 261, or 49 p.c., of 535 *An. annulipes* fed on the donors, as against 184, or 34 p.c., of 535 *An. moluccensis*. In 13 of the 14 experiments, the number of fed *An. annulipes* was greater than the number of fed *An. moluccensis*. While this evidence of the relative avidity of *An. annulipes* for human blood does not necessarily mean that its behaviour in nature is comparable, it is well known to be a vicious biter of man in the open in certain districts, and there are several observations to show that it will also enter tents for the purpose of feeding.

DISCUSSION.

These results need little discussion, though it may be added that the number of guts of *An. annulipes* with all cysts dead or showing very retarded development was 2 for *P. vivax* and 2 for *P. falciparum*, and for *An. moluccensis* these figures were 3 and 5 respectively. It is clear that *An. annulipes*, under the conditions described, is just as good an experimental host for *P. vivax* and *P. falciparum* as is *An. moluccensis*. Perhaps it is a better experimental host, but the results of experiments of this kind are notoriously so variable that it would not be justifiable to conclude so, or to test the figures mathematically for that purpose. One may fairly conclude, however, that any deficiencies which *An. annulipes* may show as a vector of malaria in nature are not due to defects in its hospitality to the parasites.

Other workers have obtained results both in accord and at variance with epidemiological findings. Thus, Swellengrebel, Shüffner, and Swellengrebel de Graaf (1919) found in the Netherlands Indies that *An. ludlowi* (*sundaicus*) was a much better experimental host than the several other species they studied, and it was by far the most important vector. On the other hand, Green and Gater (1931) showed that experimentally *An. kochi* was as readily infected as, and became more heavily infected than, *An. maculatus*, although the latter was an important vector in the coastal hills of Malaya and the former was of no practical significance at all.

It simply means that experimental findings of this kind cannot be relied on alone to determine the epidemiological importance of a species. They merely represent one, and not the most important, of the factors which must be analysed before conclusions can be drawn. In the final count, the only really satisfying evidence is that obtained from natural sporozoite rates, determined when transmission is active. Failing that, one must weigh such evidence as has been presented here, in association with evidence of abundance, feeding habits, and other biological characteristics of the insect, in order to obtain a reliable pointer to the degree of respect with which it must be treated.

It may, perhaps, be suggested in conclusion that, until this additional evidence is obtained, it would be unwise to concentrate large numbers of gametocyte carriers in places where *An. annulipes* is abundant, unless adequate protective and control measures are carried out.

SUMMARY.

It is shown that *An. annulipes* Walk. is experimentally just as hospitable to *P. vivax* and *P. falciparum* as is *An. punctulatus* var. *moluccensis* Sw. and Sw. de G. This does not indicate whether *An. annulipes* may become an important vector in nature; other, and more important, factors must be analysed before epidemiological conclusions can be drawn.

REFERENCES.

Bentley, J., Thompson, E. J. T. and Williams, R. G. (1936): Trans. Austral. Med. Cong., B.M.A., 3rd Session, Sydney, p. 261.

Green, R. and Gater, B. A. R. (1931): Bull. Inst. med. Res., F.M.S., No. 4 of 1931.

Heydon, G. A. M. (1923): Med. J. Aus., 2, p. 625.

Swellengrebel, N. H., Schüffner, W. and Swellengrebel de Graaf, J. M. H. (1919): Medek. Burgerlijk. Geneesk. Dienst. Ned.-Ind., 3, p. 1.